

STUDIES   OF   THE   PERMEABILITY  
OF   CELLS   TO   NON-ELECTROLYTES

by

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PhD Thesis,  
University of Edinburgh.

July, 1970.

DECLARATION OF ORIGINALITY

I certify that this Thesis represents my work,  
and was composed by me.





### ACKNOWLEDGEMENTS

I am indebted to my supervisor, Professor R. B. Fisher, for his help and guidance throughout my period of study. I am grateful to Dr J. A. O'Brien for his many pertinent comments.

I would like to thank Miss F. C. Wilkinson, Miss J. D. Mellor and Mrs M. Ryan for their technical assistance, and Mr M. L. G. Gardner and Mr P. F. J. Newman for taking the photographs.

I am obliged to Mrs J. S. Campbell and Mrs D. A. Phantom for typing this Thesis for me.

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## ABSTRACT

Three of the principal hypotheses which have been advanced to explain the kinetics of energy - independent permeation of cells by non-electrolytes are considered in this Thesis. Each is characterised by the step that is said to limit the rate of permeation. According to the Simple Diffusion and the Membrane Carrier Hypotheses, this step is the diffusion of the permeant across the plasma membrane; but whereas the former hypothesis postulates that diffusion is purely passive, the latter states that it is facilitated by a "carrier" located in the membrane. Conversely, the Bulk Diffusion Hypothesis supposes that the rate-limiting step is the uniformly slow diffusion of the permeant throughout the cell.

It is shown that an equation defining the time-course of permeation can be derived from each hypothesis, but that at any one concentration of extracellular non-electrolyte two of the hypothetical time-courses may be indistinguishable. However, the hypotheses predict that the time-courses change with concentration in different ways. Therefore the permeability of a cell to a non-electrolyte can be analysed in terms of the three hypotheses by determining time-courses of permeation at several concentrations.

Novel apparatus for determining the time-course of permeation of cells in suspension is described. It is a set of waisted centrifuge tubes, each of which is



divided into two compartments when a plunger is inserted into its waist. A tube is used to separate all the cells in an aliquot of the suspension from most of the suspension medium at a precisely-defined time. The fractional permeation of the cells in the aliquot is inferred from appropriate estimates made at a later time. Hence the time-course of permeation of the cells can be determined.

This is the theoretical and practical approach adopted to investigate the permeability of the human erythrocyte to five non-electrolytes: L(+) arabinose, D(+) xylose, D(+) glucose, meso-erythritol and thiourea. The investigation showed that the permeation of the aldoses and meso-erythritol is completely explicable only in terms of the Membrane Carrier Hypothesis. Whereas the carrier may have a different half-saturation constant ( $K$ ) for each anomer of the aldoses, at any one temperature between  $2.0^{\circ}\text{C}$  and  $25.5^{\circ}\text{C}$  all the anomers seem to be transferred at the same maximum rate ( $V$ ). This is consistent with the suggestion that the anomers share a common carrier. Conversely, erythritol is transferred at a maximum rate which is much less than that for the aldoses, and therefore may combine with a different carrier.

The estimates of  $K$  and  $V$  for the aldoses do not agree with those of other workers. The disagreement may reflect systematic errors in the techniques employed, real differences between the populations of erythrocytes

actually studied, or an over-simplification in the Carrier Hypothesis.

The erythrocyte appears to concentrate thiourea. Hence its permeability to this non-electrolyte could not be analysed in terms of any of the three hypotheses.

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## LIST OF SYMBOLS

The principal symbols used in this Thesis are defined below.

A	the cross-sectional area of the plasma membrane
a', b'	the coefficients of the regression of F on L: $F = b' \cdot L + a'$
a'', b''	the coefficients of the regression of L on F: $L = b'' \cdot F + a''$
$\underline{a}$ $\underline{b}$ -	alpha -, beta -
C, C <sub>J</sub>	concentration, concentration of J
c(J)	the coefficient of variation of J
D	the diffusion coefficient of intracellular permeant
E	extinction
e	the dimension of a cell of ideal shape (see p. 74)
F	$f'/t$
f	$y/x (= i/(x.w))$
f'	$i/(x.w_0)$
H <sub>J</sub>	the blank of a haemolysate for J
i	the amount of intracellular permeant
K	the half-saturation constant of the membrane carrier (see p. 47)
K', K''	the estimates of K derived from the regressions of F on L, and of L on F, respectively
k <sub>1</sub> .....k <sub>4</sub>	the rate-constants describing the operation of the membrane carrier (see pp.46, 166 ).
L	$\frac{-\ln(1-f')}{t}$
ln, log	log <sub>e</sub> , log <sub>10</sub>
m	the osmolality of the suspension medium



## List of Symbols (Cont.)

N	the number of molecules of carrier in a defined area of plasma membrane (see p.47)
n	the number of observations in a time-course
P	the permeability coefficient of the plasma membrane
p,q	the coefficients of the regression of z on t: $z = p \cdot t + q$
p <sub>0</sub>	the coefficient of the regression $z = p_0 \cdot t$
r	$k_3 / k_4$ (see p.166 )
s <sup>2</sup>	the residual variance about a regression
s(J)	the standard deviation or the standard error of J
T	transmittance
%T	per cent transmittance
t	time
V	the maximum rate of carrier-mediated permeation (see p. 47)
V', V''	the estimates of V derived from the regressions of F on L, and of L on F, respectively
v <sub>0</sub>	the volume of erythrocytes at isosmolality
var (J)	the variance of J
w	the volume of intracellular water
w <sub>0</sub>	the volume of intracellular water at isosmolality
w'	$w_0 / w$
x	the osmolality (concentration) of extracellular permeant
y	the osmolality (concentration) of intracellular permeant

## List of Symbols (cont.)

$Z_J$	the apparent increase in the concentration of J due to the presence of another compound
$z$	$Dt/e^2$ (see p.74)
$\theta$	temperature
$\mu$	micro
$\phi_J$	the osmotic coefficient of J

PART ONE

GENERAL INTRODUCTION



### Preamble

The project described in this Thesis is an investigation into the permeability of one type of cell (the human erythrocyte), to some of those non-electrolytes whose permeation does not require the expenditure of metabolic energy.

It was undertaken because, although such investigations are often used in the elucidation of cell structure and function, no completely reliable set of permeability data existed.

The human erythrocyte was chosen as the type of cell to be studied for four reasons. Firstly, its properties are relatively well-documented. Secondly, it is simple. It contains no nuclei, mitochondria, vacuoles or endoplasmic reticulum. Thirdly, the individual members of a population of erythrocytes are independent of one another, and are not contiguous with any other cell. Fourthly, a population is physiologically reproducible, in the sense that its members do not grow, divide or sporulate.

In consequence, the emphasis of this General Introduction is on the human erythrocyte. The evolution of theories of cell permeability is first outlined. This is followed by a description of some of the experimental methods used in the investigation of permeability. Finally, the organisation of the remainder of the Thesis is given.

### Theories of Cell Permeability

The theory that the cell is a basic unit of all living matter was first proposed, in 1838, by Schleiden and Schwann (Rook, 1964). Seventeen years later, Nägeli (1855) observed that the protoplasm of many plants appeared to be impermeable to the coloured anthocyanins dissolved in their vacuolar water, and suggested that the protoplasm is effectively isolated from the medium surrounding it. The idea that the permeability of a plant cell is controlled by membranes bounding its surfaces is generally attributed to Pfeffer, who seems to have adumbrated many theories of cell permeability. He suggested (Pfeffer, 1877) that the passage of water or solute through a membrane of limited permeability depends not only on the dimensions of the permeating molecule relative to those of pores in the membrane, but also on the affinity of this molecule for the membrane; and, moreover, that the rate of diffusion of permeant through water-filled pores is affected by forces acting at the water-membrane interface.

According to Collander (1959), Overton made the first systematic study of cell permeability, and then extended Pfeffer's suggestions. Overton was impressed by the similarity in permeability properties of cells or tissues which are otherwise quite different (for example: erythrocytes, muscles, root hairs and algal filaments), and noted that for many solutes, there was a positive correlation

between the permeability coefficient and the fat:water partition coefficient. Therefore he mooted, in 1898, a lipoid mechanism of permeation. It is based on the postulate that membranes are impregnated with lipids, and is that compounds traverse membranes by dissolving in these lipids. To explain the ability of cells to concentrate some solutes, Overton developed another idea of Pfeffer's by proposing, in 1902, a second, energy-dependent mechanism of penetration, which he called "adenoid". Although he stressed that the two mechanisms are fundamentally distinct from one another, many opponents of the lipoid mechanism ignored the distinction, or considered the concept of adenoid activity to smack of mere vitalism.

An alternative to Overton's lipoid mechanism was advanced by Traube, who established, between 1904 and 1913, that the rates of permeation of the members of a homologous series also increased with their degrees of adsorption at an air-water interface, and inferred that the decisive step in permeation is the adsorption of the permeant by the membrane (Davson & Danielli, 1952). Loewe supported the inference by showing that the mixture of lipids, chloroform and xylol, which Overton had used when measuring fat:water partition coefficients, formed a colloidal dispersion system rather than a true solution. Thus Overton had, in fact, been studying an adsorption phenomenon (Troshin, 1966).

However, a different concept of the structure of membranes was held by Nathansohn (1904). He supposed



that they comprise a mosaic of protein as well as lipid, with hydrophilic solutes penetrating through the protein, and hydrophobic ones penetrating through the lipid. He attributed temporal variations in the permeability of hydrophilic solutes to changes in the protein.

Hence Overton, Traube and Nathanson all felt that the permeability of a cell to a non-electrolyte is governed by interaction of the non-electrolyte with the surface membrane of the cell, but differed in their concepts of membrane structure. Another concept, that the membrane contains water-filled pores and in consequence behaves like a molecular sieve, was expounded by Ruhland & Hoffman (1925). They cited the permeability properties of a bacterium, Beggiatoa mirabilis, which were almost entirely determined by the size of the permeant molecule.

All these workers, with the possible exception of Overton, seem to have ascribed to the membrane a purely passive structure, and to have assumed that simple diffusion limits the rates of permeation of those substances which are not concentrated by the cell. They were concerned with a qualitative description of the permeability of different types of cell to different classes of compound rather than with a quantitative analysis of a particular permeability process. However, Kozawa (1914) found that isomeric hexoses and pentoses penetrated the human erythrocyte at different rates. This finding demonstrates the importance of the positions as well as the natures of the constituent groups of the permeating molecule, and cannot be explained on the basis of a passive non-specific interaction between permeant and membrane.

Ege (1919) was possibly the first to investigate the relationship between the concentration and the rate of permeation of extracellular non-electrolyte. He studied the permeability of the human erythrocyte to glucose and established two important facts. The first is that the concentration of intracellular glucose approaches that of the extracellular glucose. And the second is that the apparent permeability coefficient decreases as the concentration of extracellular glucose is increased.

Nevertheless, Collander & <sup>"</sup>Bärlund (1933), in a publication of continuing influence, presented the permeability coefficients of the giant alga, Chara ceratophylla, to a wide range of non-electrolytes, but did not demonstrate convincingly that the coefficients are independent of concentration. Moreover, although they realised that they were in fact measuring the rates at which the non-electrolytes passed through the cell wall, the plasma membrane, the protoplasm and the vacuolar membrane, they merely assumed that the plasma membrane is the rate-limiting barrier. They also measured the olive-oil:water partition coefficient of each non-electrolyte. They then showed that, to a first approximation, the logarithms of the permeability and partition coefficients are in linear relation, but that the permeability coefficients to small molecules are often greater than those predicted from the relationship. In consequence, Collander (1937) concluded that, while the plasma membrane of Chara ceratophylla seems to be composed of lipid of solvent power similar to

that of olive-oil, it also acts like a molecular sieve.

This conclusion was challenged by Danielli (1952), in the book in which he expounded his classical theory of membrane structure: the theory that the membrane is a bimolecular lipid leaflet, each surface of which is coated with protein. Danielli assumed that a non-electrolyte crosses a membrane by a process of "activated diffusion": that is, by acquiring sufficient kinetic energy to surmount the series of potential energy barriers to free diffusion presented by the membrane. He was then able to predict the relationship between a non-electrolyte's permeability coefficient, partition coefficient and chemical structure. He claimed, somewhat unjustly, that Collander & <sup>"</sup>Barlund's results conformed to his prediction, and, therefore, that the membrane may be a homogeneous lipid layer.

However, Danielli did point out that small areas of the membrane may be specifically differentiated to allow the rapid passage of glycerol. This qualification was required because Jacobs & Corson (1934) had noted that traces of cupric ions markedly retarded the influx of glycerol into the erythrocytes of some, though by no means all, species. LeFevre (1948) then suggested that, since <sup>c</sup>upric ions inhibit some enzymic reactions, enzyme-like proteins facilitate the passage of glycerol across the membrane.

LeFevre (1948) also measured the time-course of permeation of glucose into human erythrocytes at differ-



ent concentrations of extracellular glucose. He found, as had Bang & Ørskov (1937), that the apparent permeability coefficient of the cells decreased when the concentration was increased: that is, that the rate of transfer tended to a maximum. The latter authors had supposed that an increase in glucose concentration causes a "tightening" of the water-filled pores in the cell membrane through which they thought the glucose diffuses. But LeFevre showed that his time-courses could be explained if the rate of entry were determined by the difference between some limiting concentration and the concentration of intracellular glucose. He envisaged an initial combination of extracellular glucose with a saturable enzyme-like system, followed by diffusion of the bound glucose across the membrane.

Then Widdas (1951,1952) explicitly proposed a mobile carrier system to account for the transfer of glucose across the sheep placental membrane, and derived many of its kinetic properties. He pointed out that these kinetics were independent of the precise mechanism assumed for transfer. However, he mentioned that his system is consistent with the one of Lundegardh (1940), who pictured the membrane as an orientated bimolecular layer of lipid and protein, some molecules of which can acquire sufficient thermal energy to leave one interface and, by rotation of polar and non-polar groups, enter the other interface. A carrier, which is a molecule of this mobile type, can specifically combine with a molecule (or molecules) of a permeant at one interface, and release it (or them) at the other. The system is reversible, and the maximum rate at which a permeant can cross the membrane is deter-

mined by the rate at which its carriers can shuttle from interface to interface.

The kinetics of Widdas' system are much closer than are those of LeFevre's system to the observed kinetics of permeation of the human erythrocyte by glucose (LeFevre, 1962). Moreover, Widdas (1952) predicted that, if two permeants share a common carrier, the phenomenon of "counter-transport" should be demonstrable. This phenomenon was in fact first observed for glucose and xylose in rabbit erythrocytes by Park, Post, Kalman, Wright, Johnson & Morgan (1956); and, for labelled glucose and unlabelled glucose in human erythrocytes, by Rosenberg & Wilbrandt (1957).

At present, the mechanism by which non-electrolytes are specifically transported across a cell membrane, when no metabolic energy is required, and for which the term "facilitated diffusion" has been coined, is generally held to be that of a mobile carrier of the type proposed by Widdas (see, for example, Whittam, 1964, and Stein, 1967). Attempts have been made to isolate, from membranes, protein components of the monosaccharide carrier of the human erythrocyte (Bobinski & Stein, 1966), and of the lactose carrier of Escherichia coli (Jones & Kennedy, 1969).

Nevertheless, not all workers accept the mobile carrier hypothesis - or even that the rate of cellular permeation is limited by the plasma membrane. The Russian group headed by Nasanov and Troshin has developed a "sorption theory of cell permeability", in which the protoplasm plays the central role (for reviews, see

Troshin, 1961, 1966). The intracellular water is said to be bound, so that the solubilities of some compounds in it appear to be less than their solubilities in free solution; this explains why these compounds seem to be excluded from the intracellular water. Conversely, the accumulation of other compounds within the cell is due to their being bound to protoplasmic constituents, the degree of binding determining the extent of concentration. The variation with concentration of the permeability coefficient of cells to glucose is attributed to a variation with concentration of the fraction of the intracellular glucose that is bound. Unfortunately, these authors do not seem to have used their theory to predict the time-course of non-electrolyte permeation.

The "association - induction" theory of Ling (1962, 1966, 1969) bears some resemblance to the "sorption" theory. The rate of uptake is supposedly limited by the rate at which the non-electrolyte diffuses throughout the protoplasm, and not by the rate at which it crosses the plasma membrane. In this circumstance, the time-course of permeation is that of a process of uniformly slow, or "bulk", diffusion.

Fenichel & Horowitz (1963) have shown that the kinetics of efflux of several non-electrolytes from frog skeletal muscle conform to those of a bulk diffusion process. They argue that in this type of cell the cytoplasmic water forms an extensive ordered lattice stabilised by water-protein interactions, and that the existence of this lattice accounts for the observed rates

of non-electrolyte permeation. The steric specificity of permeation is determined by an obligatory interaction between the permeant and hydrogen-bonding sites at the surface of the cell. The rate of permeation could also be controlled by this interaction.

Thus there are at present two main schools of thought about the permeability of cells to non-electrolytes of low molecular weight. The proponents of the "membrane" school hold that permeability is limited by the membrane at the surface of the cell. A permeant can cross the membrane either by simple diffusion or by facilitated diffusion, and then diffuse rapidly throughout the intracellular water. Therefore the concentration of intracellular permeant is always uniform. Conversely, the supporters of the "protoplasmic" school prefer that the diffusion of the permeant throughout the protoplasm is the rate-limiting step. It follows that the concentration of intracellular permeant is inversely related to distance from the surface of the cell.

The type of cell, which members of the latter school favour in their studies, is the frog sartorius muscle. This is a syncytium with an unusual degree of internal organisation and not a simple cell like the human erythrocyte. Therefore it may have several intracellular barriers to free diffusion. Furthermore, Cope (1969), and Hazlewood, Nichols & Chamberlain (1969), have interpreted nuclear magnetic resonance spectra as meaning that the water of rat skeletal muscle exists in at least two



phases, both of which are more ordered than liquid water - although this interpretation may be open to question (Hansen & Lawson, 1970).

For the human erythrocyte, on the other hand, there is a considerable body of evidence that supports the "membrane" school. The permeation of monosaccharides is often cited as a prime example of a mechanism of facilitated diffusion, the compounds all sharing a common carrier. Thus as mentioned above, the kinetics of aldose penetration are not those of simple diffusion, but do conform to those of facilitated diffusion. Within the same concentration range, the entry of the ketoses, sorbose and fructose, appears to be by simple diffusion (LeFevre & Davies, 1951; Widdas, 1954). But as the entry of both ketoses is inhibited by glucose (LeFevre, 1954), and as the erythrocyte appears to be impermeable to m - inositol, an isomer of the monosaccharides (LeFevre & Davies, 1951; Widdas, 1954), permeation is unlikely to be by simple diffusion. Instead, it is supposed that the concentrations of these ketoses at which the carrier is half-saturated are much greater than those used in the experiments. In this circumstance, the rates of permeation by simple diffusion and facilitated diffusion are identical (see Part Two, Chapter V). LeFevre (1961a) investigated the specificity of the carrier, and concluded that the carrier interacts with the C1 conformer of a monosaccharide. However, Bowyer & Widdas (1955) found that glucose inhibits the influx of erythritol, and therefore proposed that erythritol also enters via the monosaccharide carrier.

Although the kinetics of monosaccharide permeation have received intensive study, consistent data, yielding a set of parameters which satisfactorily describes the system, have yet to be presented (Miller, 1968). The evidence for systems transporting other classes of compound is much less well-founded. For example, Hunter and his colleagues (Hunter, George & Ospina, 1965; Ospina & Hunter, 1966) have suggested, empirically, that if a compound enters a cell by simple diffusion, its rate of entry is increased by pre-treating the cell with n-butanol; and that, conversely, if such pretreatment decreases the rate of entry, some mechanism of facilitated diffusion is implied. On this tenuous basis, they have implicated a carrier in the transfer of urea, but not of thiourea, into the human erythrocyte.

Similarly, the details of the mechanism of membrane permeation by simple diffusion are subject to dispute. Solomon and his co-workers (Goldstein & Solomon, 1960; Solomon, 1960b) believe that the membrane contains pores, which have an "equivalent pore radius" of about 400 pm (i.e.  $400 \times 10^{-12}$  m). They contend that small molecules can diffuse through these water-filled pores, while larger molecules pass through the lipid of the membrane.

On the other hand, Zwolinski, Eyring & Reese (1949), Stein (1967), and Lieb & Stein (1969) have refined Danielli's (1952) analysis of the properties expected for a mechanism based on a membrane that presents a continuous barrier of lipid and protein through which a permeant must diffuse. According to Lieb & Stein, when

permeation occurs in the absence of the bulk flow of water, the observed and predicted permeability coefficients are in good agreement, given that the membrane behaves as though it were a network of polymers. There is no need to postulate the existence of pores. Unfortunately, the permeability coefficients to most of the compounds they considered were measured before 1940, by either Collander or Jacobs and their co-workers, usually at only one concentration of extracellular permeant, and on the assumption that Fick's Law was being obeyed. Therefore it is possible that some, if not all, of these compounds are like the ketoses, in that they enter the cell by mechanisms of facilitated diffusion that are only appreciably saturated at concentrations higher than those actually used.

### Methods Used to Study Cell Permeability

It is obvious that any attempt to resolve the conflicting views on the mechanisms of membrane permeation must be based on accurate data. A theoretical objection to the early studies - that is, those of Collander & "Bärlund (1933), and of Jacobs, Glassman & Parpart (1935) - is mentioned above. These studies can also be criticised on practical grounds. Collander & "Bärlund estimated the concentration of permeant in the vacuolar water of an experimental cell at a particular time by comparing its dichromate titre with that of the vacuolar water of a suitable control cell. These estimates are imprecise, may well be inaccurate, and can only be made when permeation occurs slowly. Jacobs et al. measured the time required for the haemolysis of 75% of the erythrocytes suspended in a solution of permeant. Short times are difficult to measure accurately, and, in any case, the work of Staverman (1952) has demonstrated that the theory underlying Jacobs et al.'s calculation of permeability coefficients is unsound. The values they deduced for rapidly-permeating compounds contain gross errors (Stein, 1967).

Later studies of erythrocyte permeability have been designed to find the time-course of permeation, given that extracellular and intracellular non-electrolyte equilibrate with one another fairly rapidly. For example, when glucose-free erythrocytes are suspended in a large volume of isosmolal medium, containing 10 mM glucose at 25°C,



the concentration of intracellular glucose reaches 9 mM after about 40 s. This implies that any method that is used to determine such a time-course must either give a continuous record of the relative concentrations of extracellular and intracellular permeant, or unequivocally terminate permeation at a particular instant so that the relative concentrations at that instant can be determined later.

A popular technique which may fulfil the former alternative was invented by Ørskov (1935), and exploited by LeFevre (1948) and Widdas (1954). Its principle is the continuous measurement throughout the permeation process of the amount of light transmitted by a dilute suspension of erythrocytes. Two assumptions are usually made:

- (i) That the amount of light transmitted by the suspension is linearly related to the mean volume of the erythrocytes.
- (ii) That water enters the cell much more rapidly than does permeant. In consequence, the erythrocytes are always in osmotic equilibrium with the extracellular medium, and changes in the volume of the cells are due only to changes in the concentration gradient of permeant.

In a typical experiment, erythrocytes which have been equilibrated with permeant are injected into a suitable medium of different permeant concentration. This disturbs the osmotic equilibrium of the cells, so that their volume changes "instantaneously". The slower return of the volume to that of isosmolality, caused by re-equili-

bration of permeant, is then recorded.

The technique may be criticised on several grounds.

For example:

- (i) The first of the above assumptions must be justified. Kay (1964) has pointed out that the precise relationship between the two variables depends on exactly how, and at what wave-length, the amount of light transmitted by the suspension is measured; on the fraction of the secondary scattered light accepted by the detectors; and on the haematocrit of the cell suspension.
- (ii) The second fundamental assumption, that changes in the volume of the cells are due only to changes in the concentration gradient of permeant, is not entirely true. This point is elaborated in Chapter II of Part Two.
- (iii) It may be difficult to define, from the record of the amount of light transmitted by the suspension, the instant at which permeation began (see, for example, Figure 1 of Miller (1965a)). A small error in zero time might cause appreciable errors in the parameters of permeation estimated from the record.

The technique has the appealing advantage of being much less laborious than those dependent on the chemical estimation of permeant concentrations at known times, and can be used with any compound that does not alter the physical characteristics of the cell. On the other hand, it can only measure a rate of net flux of permeant. If, for example, the rate of unidirectional flux of a permeant at concentration equilibrium is required, another method, such as one of those described below, must be employed.

A widely-used way of studying monosaccharide permeation in the human erythrocyte was introduced by Britton

(1956,1964), and by LeFevre & McGinniss (1960). Its basis is an observation of LeFevre's (1948), that 1 mM mercuric chloride inhibited glucose transfer. In practice, inhibition is achieved by adding 1 vol. of the cell suspension to 5 or 10 vol. of a mercuric chloride - sodium chloride solution at 0°C. The validity of the method clearly depends on the assumption that mercuric chloride completely and instantaneously stops monosaccharide transfer. Britton (1964) reported that although he was unable to detect any leakage of intracellular glucose from "inhibited" cells containing 133 mM glucose, about 5% of the intracellular glucose was lost from erythrocytes which contained 16.7 mM glucose on inhibition. Therefore it is encouraging that Miller (1965a) found no significant difference between the parameters characterising the facilitated diffusion of galactose and mannose, when he estimated them by both the photometric and the mercuric chloride methods.

The mercuric chloride method works for monosaccharides, but may not be applicable to other permeants. A more general procedure was devised by Mawe & Hempling (1965). They used a syringe, fitted with a Millipore filter, to remove a sample of extracellular medium from a dilute suspension of erythrocytes at a precise time. This procedure is of limited usefulness, because the contents of the cells themselves cannot be estimated. In consequence, only the efflux of permeant can be monitored. Moreover, if a significant fraction of the cells is haemo-

lysed when a sample of extracellular medium is withdrawn, the concentration of permeant in this sample may be significantly increased.

None of these methods for determining kinetics of permeation is entirely satisfactory. A novel method, described in detail in Part Three, has therefore been developed. It is simple in principle. The fundamental pieces of equipment are a waisted centrifuge tube, and a plunger which is used to divide the tube, at its waist, into an upper and a lower compartment. The erythrocyte suspension is incubated with permeant in the centrifuge tube for the desired length of time. Rapid centrifugation then transfers all the cells into the part of the tube below the waist, and the plunger is inserted. Thus the upper compartment contains extracellular medium only, while the lower one holds both extracellular medium and all the cells originally present in the suspension. The compositions of both the extracellular and the intracellular media, at the moment that the insertion of the plunger "divided" the tube, are easily calculated from those of the upper and lower compartments.

These centrifuge tubes have been used in an attempt to determine the kinetics of permeation of the compounds listed below. The kinetics could have been inferred from the time-courses of permeation, either when the cell volume varied continuously, or when it was constant. Since the theoretical time-course of a process limited by a step of bulk-diffusion is easily derivable only for a cell of ideal shape and constant volume, the measure-



ment of time-courses at constant cell volume would seem to have been necessary. However, this approach has two flaws. Firstly, it means studying the movement of labelled permeant between media whose total concentrations of permeant are equal. Such movements would be misinterpreted if the permeant exhibited "exchange diffusion" across the membrane: that is, the obligatory exchange of a molecule of intracellular permeant for one of extracellular permeant. Secondly, if there is no net transfer of water between the extracellular and intracellular media, unstirred layers may be present at the surfaces of the cells. The presence of these layers could introduce serious errors into an observed time-course. Because most experimental evidence suggests that the rate of permeation of the human erythrocyte is limited by the membrane, and not by the protoplasm, the experiments reported in this Thesis were initially designed to provide accurate data which could be analysed in terms of membrane - limited mechanisms. The time-courses were therefore measured in conditions in which the cell volume varied.

The compounds examined are D(+) glucose, L(+) arabinose, D(+) xylose, meso-erythritol and thiourea. The first three, which are alleged to share the same system of facilitated diffusion, were chosen because this system requires further characterisation. Erythritol was chosen because it, too, may use the "monosaccharide" system. And thiourea was chosen because it is often assumed, on the basis of tenuous evidence, to permeate by simple diffusion.

## Organisation of Thesis

This Thesis comprises five Parts and three Appendices. In Part Two ("Theory"), the theoretical time-courses of processes limited by simple diffusion across a thin membrane, by a mobile carrier located in this membrane, and by slow diffusion throughout the intracellular water ("bulk diffusion"), are derived. Methods for comparing them with an observed time-course are described. Finally, the theoretical time-courses are compared with one another. The comparisons show that, in certain circumstances, the time-courses predicted from the Simple Diffusion and the Membrane Carrier Hypotheses, and from the Membrane Carrier and the Bulk Diffusion Hypotheses, may be indistinguishable.

Part Three is entitled "Experimental Methods and Materials". It includes a description of the erythrocyte preparation studied, of the design and use of the waisted centrifuge tubes, and of the procedures by which the permeants were estimated.

The experimental results obtained are given in Part Four. These indicate that the three aldoses do, indeed, permeate via the same system of facilitated diffusion, and that the system appears to distinguish between alpha - and beta - glucose. <sup>\*\*</sup> The effect of temperature on the rate of permeation permits further characterisation of the

\*\* In the rest of this Thesis, alpha - and beta- glucose are denoted by a- and b- glucose, respectively.

system. On the other hand, erythritol appears to permeate via a different system. Unfortunately, no useful permeation data on thiourea was obtained. The reasons for this failure are discussed.

The Thesis concludes with Part Five, a General Discussion. The significance of the work described in the preceding Parts is assessed in terms of current theories of cell permeability, and further experiments are suggested.

PART   TWO

THEORY



This project is an investigation into the ways in which non-electrolytes permeate the human erythrocyte. Three hypotheses of non-electrolyte permeation merited consideration: they are outlined in the General Introduction, and are elaborated in the rest of this Part. Each is characterised, as follows, by a step that limits the rate of permeation:

- (1) The Simple Diffusion Hypothesis: the rate-limiting step is the diffusion of permeant through the plasma membrane.
- (2) The Membrane Carrier Hypothesis: the rate-limiting step is the carrier-mediated transfer of permeant through the plasma membrane.
- (3) The Bulk Diffusion Hypothesis: the rate-limiting step is the diffusion of permeant throughout the intracellular medium.

Each rate-limiting step is, in turn, characterised by two parameters which summarise its kinetics and which are constant for a given non-electrolyte at a fixed temperature. The parameters that characterise a step of Simple Diffusion are  $P$ , the permeability coefficient of the membrane to the non-electrolyte, and  $A$ , the cross-sectional area of the membrane. A membrane carrier, in its simplest form, is characterised by  $K$  and  $V$ , which are similar to, though not identical with, the half-saturation constant and the maximum velocity, respectively, of an enzyme-catalysed reaction. Finally, Bulk Diffusion is characterised by  $D$ , the diffusion coefficient of intracellular non-electrolyte, and  $e$ , which specifies the size

of the cell.

A general equation which defines the time-course of permeation ("the permeation equation") can be derived from each of the hypotheses. The exact form of a time-course defined by one of these equations depends on the nature of the rate-limiting step, on the values of the characteristic parameters, and on the experimental conditions. It is therefore possible to test, as follows, whether or not the permeability of a cell is explicable in terms of a particular hypothesis. A time-course of permeation is determined in known experimental conditions. The permeation equation is then fitted to the observed time-course: that is, the values of the parameters that minimise the sum of the squares of the differences between the hypothetical and the observed time-courses are calculated. If the differences can be attributed to experimental error, the hypothesis seems to explain the permeation by the non-electrolyte. Furthermore, the best-fit estimates of the parameters thought to characterise the hypothetical rate-limiting step appear to have meaning.

This test does not necessarily permit the conclusion that the observed time-course cannot be explained by another hypothesis, because, as shown in Chapter V of this Part, the hypothetical time-courses themselves may be indistinguishable from one another. The conclusion can only be drawn after the observed kinetics of permeation have satisfied further criteria.

One such criterion is based on the apparent values of the parameters characterising the step that is supposed to be rate-limiting. These are constant for a given non-electrolyte at a fixed temperature, and are independent of the concentration of extracellular non-electrolyte. Since the test described above requires that the values of the parameters be estimated, this criterion can be used to increase its rigour. If the hypothetical and observed time-courses, determined at several concentrations of extracellular permeant, are always indistinguishable from one another, and if the several sets of estimates of the parameters do not differ, the hypothesis appears to account for the permeation by the non-electrolyte.

It is also shown in Chapter V that the original test, fortified by the inclusion of this criterion, allows the three hypothetical time-courses to be resolved. In consequence, its use reveals not only which of the three hypotheses best explains an observed rate of permeation, but also the values of the parameters characterising the rate-limiting step.

There are five more Chapters in this Part. Chapters II, III and IV are concerned, respectively, with the hypotheses of Simple Diffusion, the Simple Membrane Carrier, and Bulk Diffusion. In each, a permeation equation is derived, and some of the approximations made in the derivation are discussed. A method for fitting a permeation equation to an observed time-course, for assessing the goodness of the fit, and for estimating the

characteristic parameters, is described and then evaluated. In Chapter V, the hypothetical time-courses are compared with one another, and the test for their resolution is justified. The closing Chapter is a short summary.



## Chapter II THE SIMPLE DIFFUSION HYPOTHESIS

### Section 1

### Introduction

Jacobs (1952) has summarised the Simple Diffusion Hypothesis, and the kinetics of permeation derived from it. He assumed that the rate of net permeation of a mass of cells by a non-electrolyte obeys Fick's Law of Diffusion:

$$\frac{di}{dt} = w_o \cdot PA \cdot (x-y) \quad (2.01)$$

where  $i$  = the number of osmoles of intracellular non-electrolyte;

$t$  = the time;

$w_o$  = the volume of intracellular water when the mass of cells is in osmotic equilibrium with a medium of osmolality  $m$ ;

$P$  = the permeability coefficient of the membrane;

$A$  = the cross-sectional area of the membrane of the mass of cells;

$x, y$  = the osmolalities of the extracellular and intracellular non-electrolyte, respectively.

The equation only holds in certain circumstances. It is important that the permeability of the cell to the non-electrolyte be small compared with that to water. This implies that the non-electrolyte enters the cell by diffusion only, rather than by a combination of diffusional and bulk transfer; that its reflection coefficient is unity, so that it exerts its full osmotic effect across the membrane; and that it is uniformly distributed in the

intracellular and extracellular media. Furthermore, the membrane must be so thin, that there is a constant gradient of non-electrolyte at all points between its two surfaces. If the non-electrolyte and water enter the cell at comparable rates, some other theoretical approach is required: for example, that of Kedem & Katchalsky (1958, 1961), which is founded on non-equilibrium thermodynamics. However, all the non-electrolytes which have been investigated seem to cross the erythrocyte membrane much less rapidly than does water. Therefore the kinetics of permeation are given by equation (2.01).

This Chapter is divided into five Sections. A permeation equation is derived in Section 2. In Section 3, some of the assumptions underlying the derivations are examined and sustained. Section 4 includes a description of a method for fitting the equation to a time-course, for assessing the goodness of the fit, and for estimating PA. The last Section comprises a Summary.

## Section 2      The Permeation Equation

The equation for the time-course of permeation is derived by integrating equation (2.01):

$$\frac{di}{dt} = w_o \cdot PA \cdot (x-y) \quad (2.01)$$

The integration performed below is for an experiment in which P, A and x are constant, and y is a function of i. In this experiment, a mass of cells containing a volume  $w_o$  of intracellular water is initially in osmotic equilibrium with an isosmolal medium of osmolality m which is free of permeant. At time zero permeant is added to this medium, making its total osmolality  $(m+x)$ . At time t, when i osmoles of permeant have entered the cells, the volume of intracellular water, w, is:

$$w = \frac{m \cdot w_o + i}{m+x} \quad (2.02)$$

provided that:

- (1) the intracellular and extracellular media have equal osmolalities;
- (2) the total number of osmoles of intracellular material, except permeant, is constant;
- (3) the intracellular water is uniformly accessible to all solutes;
- (4) the total osmolality of the extracellular medium, and the osmolality of the permeant in it, do not change.

The extent to which these provisos are fulfilled by the human erythrocyte is discussed in the next Section of this Chapter.

At time  $t$ , the osmolality of intracellular permeant,  $y$ , is:

$$y = \frac{i}{w} = \frac{m+x}{m \cdot w_o + i} \cdot i \quad (2.03)$$

Substituting for  $y$  in equation (2.01):

$$\frac{di}{dt} = w_o \cdot PA \left( x - \frac{m+x}{m \cdot w_o + i} \cdot i \right) \quad (2.04)$$

Rearrangement and integration of equation (2.04) gives:

$$- (i/w_o) - (m+x) \cdot \ln(1 - (i/x \cdot w_o)) = m \cdot PA \cdot t \quad **$$

provided that  $PA$  is constant, and that  $i = 0$  when  $t = 0$ .

If  $f' = i/(x \cdot w_o)$ , this becomes:

$$-x \cdot f' - (m+x) \cdot \ln(1-f') = m \cdot PA \cdot t \quad (2.05)$$

The time-course of permeation is defined by the permeation equation, 2.05. The variable,  $f'$ , which is a measure of the amount of intracellular permeant at time  $t$ , is not equal to the ratio of the osmolalities of intracellular and extracellular permeant at this time (that is, to  $y/x$ ). It is, however, related to this ratio. From equation (2.03):

$$\frac{y}{x} = \frac{m+x}{m \cdot w_o + i} \cdot \frac{i}{x}$$

so that, putting  $f = y/x$ ,

$$f = \frac{(m+x) \cdot f'}{m+x \cdot f'} \quad (2.06)$$

\*\* In this Thesis, "ln" denotes " $\log_e$ ", and "log" denotes " $\log_{10}$ ".



### Section 3 The Applicability of the Permeation Equation to the Human Erythrocyte

The permeation equation, 2.05, is only true for an experimental system that obeys the provisos mentioned in the previous Section. The extent to which the human erythrocyte fulfils these provisos is the subject of the remainder of this Section.

#### 3.01 The Equality of Intracellular and Extracellular Osmotic Pressures

It is generally assumed that there is no gradient of osmotic pressure across the membrane of a human erythrocyte of constant volume. Dick (1959), and Robinson (1960), have reviewed some of the attempts formally to prove the truth of the assumption. They both opined that, although it is difficult to measure an intracellular osmotic pressure with accuracy, departures from osmotic equilibrium are trivial. Nevertheless, Rand & Burton (1964a) have interpreted their investigations into mechanical properties of the membrane of the human erythrocyte to mean that there is a gradient of hydrostatic pressure, and hence of osmotic pressure, across this membrane. They have suggested (1964b) that these pressure differences are related to an active transfer of  $\text{Na}^+$  and  $\text{K}^+$ . Since the postulated pressure differences for a cell in isosmolal or hyposmolal solution are only equivalent to 2 or 3 mm of water, they need not be entertained further. For the same reason, any pressure differences due to the packing of erythrocytes by the centri-

fugal forces used in this study are discounted.

It is evident that the opinion of Dick and Robinson might not apply to an erythrocyte whose volume was changing - particularly if the rate of net permeation of non-electrolyte were comparable with that of water. However, it is pointed out in the preceding Section that in the latter circumstance equation (2.01) does not hold, and some other theoretical treatment is required.

### 3.02 The Constancy of the Number of Osmoles of Intracellular Material

The assumption is vitiated if at any time during an experiment:

- (i) the mean osmotic coefficient of the intracellular material varies;
- (ii) there is a net transfer of osmotically-active particles, other than those of permeant, between the intracellular and extracellular media;
- (iii) the number of intracellular osmotically-active particles changes.

The last of these possibilities can be dismissed at once because, over the period of time for which permeation was normally observed, there was no detectable change in the volume of a mass of erythrocytes incubated in suspension medium alone (see Table 3.02, facing p.100). The first two possibilities are discussed in turn below.

At isosmolality, each litre of the intracellular water of the human erythrocytes used in this study held about 160 mequiv. of  $\text{Na}^+$  and  $\text{K}^+$ , 100 mequiv. of  $\text{Cl}^-$ , 6 mmol of inorganic phosphate, and 7.6 mmol of haemo-

globin (see Table 3.01, facing p. 98). Since these compounds generated more than 90% of the intracellular osmotic pressure, it is the concentration-dependence of their osmotic coefficients which is important.

Robinson & Stokes (1955) have tabulated the concentrations and osmotic coefficients of aqueous solutions of several pure inorganic electrolytes. For example, for KCl at 25°C, they list the osmotic coefficient,  $\phi_{\text{KCl}}$ , at three concentrations, (KCl), between 0.2 and 0.6 g-ion/l. A second-order regression of  $\phi_{\text{KCl}}$  on (KCl) was fitted to their data:

$$\phi_{\text{KCl}} = 0.9479 - 0.1123 (\text{KCl}) + 0.0874 (\text{KCl})^2$$

As there are only inadequate data on the osmotic coefficients of electrolytes in multi-component solutions, and as  $\text{K}^+$  and  $\text{Cl}^-$  are the inorganic ions present in the intracellular water in the greatest concentrations, the mean osmotic coefficient of all the intracellular inorganic ions,  $\phi_s$ , is equated to that of a solution of KCl of the same total concentration:

$$\phi_s = 0.9479 - 0.1123 (s) + 0.0874 (s)^2 \quad (2.07)$$

(s), the concentration of ions in g-ion/l, is roughly 0.270 at isosmolality. Hence, when the volume of intracellular water changes from its isosmolal value,  $w_o$ , to any other value, w, (s) becomes  $0.270 \cdot (w_o/w)$  g-ion/l. If  $w'$  is substituted for  $(w_o/w)$ , equation (2.07) can be re-written as:

$$\phi_s = 0.9479 - 0.0309 w' + 0.0066 w'^2 \quad (2.08)$$

Adair (1929), and McConaghey & Maizels (1961), have studied the way in which the osmotic coefficient of

haemoglobin depends on concentration. Adair's data are for pure solutions containing from 2 to 5 mmol of haemoglobin per kg of water, and are therefore best used to describe the osmotic behaviour of the protein in swollen erythrocytes. Dick & Lowenstein (1958) summarised the data by the equation:

$$\phi_{\text{Hb}} = 1 + 0.106 (\text{Hb}) + 0.020 (\text{Hb})^2$$

where  $\phi_{\text{Hb}}$  is the osmotic coefficient of a haemoglobin solution of milli molal concentration (Hb).

McConaghey & Maizels worked with solutions of between 5 and 20 mmol of haemoglobin per kg of water, and variable amounts of salt. Therefore their data refer best to the haemoglobin in shrunken erythrocytes. The first-order regression fitting the data is:

$$\phi_{\text{Hb}} = 0.829 (\text{Hb}) - 2.920 \quad (2.09)$$

and the addition of a term in  $(\text{Hb})^2$  does not improve the fit significantly (analysis of variance;  $P > 0.05$ ). Since, at isosmolality, an erythrocyte contains about 7.6 mmol of haemoglobin per kg of water, equation (2.09) can be transformed to:

$$\phi_{\text{Hb}} = 6.30 w' - 2.92$$

The mean osmotic coefficient of the material in shrunken erythrocytes,  $\phi_{\text{M}}$ , is obtained by combining (s),  $\phi_{\text{s}}$ , (Hb) and  $\phi_{\text{Hb}}$ :

$$\begin{aligned} \phi_{\text{M}} &= \frac{(s) \cdot \phi_{\text{s}} + (\text{Hb}) \cdot \phi_{\text{Hb}}}{(s) + (\text{Hb})} \\ &= \frac{234 + 39.5 w' + 1.8 w'^2}{277.6} \end{aligned}$$



Thus, when the mass of cells is in osmotic equilibrium with medium of osmolality  $m$  (that is, in the terms of the "experiment" considered in Section 2 of this Chapter, when  $t=0$  and  $w=w_0$ ), the total number of osmoles of intracellular material,  $M$ , is

$$M = (0.2340 + 0.0395 + 0.0018) \cdot w_0 = m \cdot w_0 \quad (2.10)$$

At time  $t$ , when the content of permeant and the volume of intracellular water are  $i$  osmoles and  $w$ , respectively:

$$M = (0.2340 + 0.0395 w' + 0.0018 w'^2) \cdot w_0 + i$$

Substituting  $(m \cdot w_0)$  from equation (2.10):

$$M = m \cdot w_0 + i + 0.0395 \cdot w_0 \cdot (w' - 1) + 0.0018 \cdot w_0 \cdot (w'^2 - 1)$$

Hence  $w$  is given, not by equation (2.02):

$$w = \frac{m \cdot w_0 + i}{m + x} \quad (2.02)$$

but by equation (2.11):

$$w = \frac{m \cdot w_0 + i + 0.0395 \cdot w_0 \cdot (w' - 1) + 0.0018 \cdot w_0 \cdot (w'^2 - 1)}{m + x} \quad (2.11)$$

However, the outcome of an examination of the second of the three possibilities, listed at the beginning of this sub-section, is that equation (2.11) requires further modification. For example, consider a human erythrocyte suspended in a large volume of isosmolal NaCl - Na phosphate medium, pH 7.4. When permeant is added to the medium, the erythrocyte shrinks "instantaneously", and then swells to its initial volume as the permeant enters it. In this experiment, the only other osmotically-important solute which can enter or leave the cell is  $Cl^-$  (Jacobs & Stewart, 1947). Now, it is well known (Whittam,

1964) that the distribution across the erythrocyte membrane of some anions conforms to a Gibbs-Donnan equilibrium; in this experiment:

$$\frac{(\text{OH}^-)_e}{(\text{OH}^-)_i} = \frac{(\text{Cl}^-)_e}{(\text{Cl}^-)_i} = g \quad (2.12)$$

where e and i refer the extracellular and intracellular anions, respectively. When the cell shrinks,  $(\text{OH}^-)_e$  and  $(\text{Cl}^-)_e$  remain constant.  $(\text{OH}^-)_i$  and  $(\text{Cl}^-)_i$  increase - but, because of the buffering capacity of intracellular haemoglobin, in different proportions. An outward diffusion of  $\text{Cl}^-$  ions, balanced by an inward diffusion of an equal number of  $\text{OH}^-$  ions, virtually instantaneously restores the Gibbs-Donnan equilibrium. It also causes a net decrease in the total number of diffusible intracellular anions.

The magnitude of the decrease can be estimated as follows. Suppose that, at isosmolality, the concentrations of extracellular and intracellular anions are  $(\text{OH}^-)_o$ ,  $(\text{Cl}^-)_o$ , and  $(\text{OH}^-)_1$ ,  $(\text{Cl}^-)_1$  g-ion/l respectively, and that the concentration of intracellular haemoglobin is  $(\text{Hb})$  mol/l. Suppose, also, that the change in the volume of intracellular water from  $w_o$  litres to  $w$  litres is accompanied by an efflux of  $n$  g-ion of  $\text{Cl}^-$ , and an influx of  $n$  g-ion of  $\text{OH}^-$ . The concentration of intracellular  $\text{Cl}^-$ ,  $(\text{Cl}^-)_2$ , is given by:

$$w \cdot (\text{Cl}^-)_2 = w_o \cdot (\text{Cl}^-)_1 - n \quad (2.13)$$

The influx of  $\text{OH}^-$  raises the intracellular pH to  $\text{pH}_2$  from its initial value of  $\text{pH}_1$ . Since the addition of 2.54 g-ion of  $\text{H}^+$  to 1 mol of haemoglobin in a solution whose pH

is within the physiological range lowers the pH by 1 unit (Davenport, 1958); and since haemoglobin supplies 90% of the total buffering capacity of the erythrocyte (Harris & Maizels, 1952):

$$pH_2 - pH_1 = n / (2.54 \cdot (Hb) \cdot w_o) \quad (2.14)$$

Equation (2.12) can be used to derive another expression for  $(pH_2 - pH_1)$ :

$$\text{Initially,} \quad \frac{(OH^-)_o}{(OH^-)_1} = \frac{(Cl^-)_o}{(Cl^-)_1} = g_1 \quad (2.15)$$

$$\text{so that} \quad pH_o - pH_1 = \log \frac{(Cl^-)_o}{(Cl^-)_1} = \log g_1 \quad (2.16)$$

$$\text{Similarly,} \quad pH_o - pH_2 = \log \frac{(Cl^-)_o}{(Cl^-)_2} \quad (2.17)$$

Thus, combining equations (2.13), (2.16) and (2.17):

$$pH_2 - pH_1 = \log g_1 - \log \left( \frac{w \cdot (Cl^-)_o}{w_o \cdot (Cl^-)_1 - n} \right)$$

Substituting  $(Cl^-)_o$  from equation (2.15):

$$\begin{aligned} pH_2 - pH_1 &= \log g_1 + \log \left( \frac{w_o \cdot (Cl^-)_1 - n}{w \cdot g_1 \cdot (Cl^-)_1} \right) \\ &= \log w' + \log (1 - n / (w_o \cdot (Cl^-)_1)) \end{aligned} \quad (2.18)$$

If  $n$  is small compared with  $w_o \cdot (Cl^-)_1$  - that is, if only a small fraction of the intracellular  $Cl^-$  diffuses out of the cell - equation (2.18) approximates to:

$$pH_2 - pH_1 = \log w' - n / (\ln 10 \cdot w_o \cdot (Cl^-)_1)$$

Substituting for  $(pH_2 - pH_1)$  in equation (2.14), and rearranging:

$$n = w_o \cdot \ln w' \cdot \frac{2.54 \cdot (Hb) \cdot (Cl^-)_1}{2.54 \cdot (Hb) + \ln 10 \cdot (Cl^-)_1}$$

For the cells in this experiment,  $(Hb) = 7.6 \text{ mM}$ ,  $(Cl^-)_1 = 100 \text{ mg-ion/l}$ , and, from equation (2.08),  $\phi_{Cl^-} = 0.92$ .

Hence:

$$n = 7.14 \cdot w_o \cdot \ln w' \quad \text{milliosmoles}$$

Equation (2.11) is thus modified to:

$$w = \frac{m \cdot w_o + i + 0.0395 \cdot w_o \cdot (w' - 1) + 0.0018 \cdot w_o \cdot (w'^2 - 1) - 0.0071 \cdot w_o \cdot \ln w'}{m + x}$$

However, when  $1.0 \geq w \geq 0.5$  :

$$0.0018 \cdot (w'^2 - 1) - 0.0071 \cdot \ln w' \ll 0.0395 \cdot (w' - 1)$$

so that:

$$w = \frac{m \cdot w_o + i + 0.0395 \cdot w_o \cdot (w' - 1)}{m + x} \quad (2.19)$$

Solving equation (2.19) for  $w$ , which must be positive:

$$w = \frac{((m - 0.0395) \cdot w_o + i) + ((m - 0.0395) \cdot w_o + i)^2 + 0.158(m + x) \cdot w_o^2}{2(m + x)} \quad (2.20)$$

The permeation equation was derived on the simplifying assumption that  $w$  is given by equation (2.02). If, instead, equation (2.20) is used to find  $w$ , equation (2.01) cannot be integrated algebraically, and an explicit permeation equation cannot be derived. But it is possible to integrate equation (2.01) numerically, and therefore to assess the magnitude of the error introduced by using the simpler of the two expressions for  $w$ .



The numerical integration has not been performed, because the kinetics of permeation of the non-electrolytes investigated do not even approximate to those of Simple Diffusion (See Part Four). However, it is shown in Chapter III, Section 3, that the simplification makes little difference to a time-course predicted from the Simple Membrane Carrier Hypothesis. Since the Simple Diffusion and the Membrane Carrier permeation equations are similar to one another, it seems reasonable to infer that the use of the simplification does not appreciably alter a time-course of Simple Diffusion.

### 3.03 The Homogeneity of the Intracellular Water

Only about 80% of the water in a human erythrocyte appears to respond to changes in extracellular osmolality (Ponder, 1948). In consequence, it has often been suggested that the remaining 20% is bound to proteins, and unavailable to other intracellular solutes (see, for example, LeFevre, 1964; Savitz, Sidel & Solomon, 1964).

However, a considerable body of evidence makes the suggestion untenable. For example, it is easy to calculate that if all the diffusible compounds known to be in the cell were dissolved in 80% of the intracellular water, their total osmolality would be significantly greater than that of the extracellular medium. The difference could be due to these compounds' being bound, to their having reduced activity coefficients, or to a gradient of hydrostatic pressure across the membrane.

But there are no observations to support these explanations. Furthermore, Gary Bobo (1967) confirmed the conclusion of Miller (1964), that glucose permeates all the intracellular water. And Cook (1967) found that  $\text{Cl}^-$  was excluded by not more than 3% of this water. Therefore it is reasonable to ignore the possibility of "bound" or "non-solvent" water: especially as the "anomalous" osmotic properties of the erythrocyte can be accounted for in other ways.

Thus Hendry (1954) claimed that the haematocrit value of a suspension of swollen erythrocytes is artificially reduced by compression of the cells. In consequence, the volume of the cells is underestimated. Dick (1966, 1969) has maintained that the variation with concentration of the osmotic coefficient of haemoglobin is sufficient to explain the observed relationship of intracellular volume to extracellular osmolality, and that there is no need to invoke the concentration-dependence of the net charge on this protein which was proposed by Gary Bobo & Solomon (1968). Finally, Cook (1967) showed that when erythrocytes were suspended in a hyperosmolal solution of NaCl the number of intracellular ions increased.

The preceding sub-section includes a discussion of how the permeation equation is affected by variations with cell volume of the osmotic coefficient of haemoglobin and of the number of intracellular ions. Hence the anomalous osmotic behaviour of the erythrocyte needs no further consideration.

### 3.04 The Constancy of the Osmolality of the Extracellular Medium

It is evident that in any real experiment the osmolality of the extracellular medium, and of the permeant dissolved in it, change with time. Equation (2.01):  
that is

$$\frac{di}{dt} = w_o \cdot PA \cdot (x-y) \quad (2.01)$$

can be modified to allow for these changes.

Suppose that at time zero I osmoles of permeant are added to the extracellular medium, which is of osmolality m and contains W kg of water. At time t, when i osmoles of permeant have entered the cells, the total osmolality of the extracellular medium,  $m_e$ , is

$$m_e = \frac{m \cdot W + I - i}{W + w_o - w}$$

It is inferred above (p.38) that little error is introduced by assuming that the intracellular osmolality,  $m_i$ , is given by

$$m_i = (m \cdot w_o + i) / w$$

Since  $m_e = m_i$ , it follows that

$$w = \frac{(W + w_o) \cdot (m \cdot w_o + i)}{m \cdot (W + w_o) + I} \quad (2.21)$$

The osmolality of extracellular permeant, x, is

$$x = \frac{I - i}{W + w_o - w} \quad (2.22)$$

and the osmolality of intracellular permeant, y, is

$$y = \frac{i}{w}$$

If  $x$  and  $y$  are replaced by these expressions, equation (2.01) cannot be integrated algebraically. However, it can be integrated numerically, so that it is possible to establish whether or not the variations in  $m_e$  and  $x$  significantly alter a particular time-course.

The numerical integration has not been carried out. But it is shown in Chapter III, Section 3, that the variations in  $m_e$  and  $x$  do not alter much a time-course predicted from the Simple Membrane Carrier Hypothesis. The same is presumed to be true for one from the Simple Diffusion Hypothesis.

### 3.05      The Constancy of $P$ and $A$

According to Jacobs (1932), the surface area of the human erythrocyte remains constant for even quite large increases in cell volume. There do not appear to have been any studies of the way in which the surface area changes when the cell shrinks. Hence it is difficult to speculate about the variation of  $A$  with  $w$ . The easy solution is to assume that, since there is no evidence to the contrary,  $A$  is independent of  $w$ .

However, there is some evidence that  $P$  may depend on  $w$ . Solomon (1960a) reported that there is an inverse relation between the two variables when the permeant is water. On the other hand, Miller (1964) found that the permeability of erythrocytes to fructose increased when the cells were suspended in hyperosmolar NaCl, but did not alter when some of the NaCl was replaced by an equi-



osmolal amount of lactose. Thus it seems fair to make the initial assumption that, in the present investigation,  $P$  is independent of  $w$ .

### 3.06 Conclusion

The permeation equation, 2.05, is only an approximation. In its derivation, no account is taken either of the dependence on concentration of the osmotic coefficient of haemoglobin or of changes in the osmolality of extracellular non-electrolyte. An explicit equation cannot be derived if these variables are included. But it seems that their exclusion makes little difference to a time-course predicted from the hypothesis.



## Section 4 Fitting the Permeation Equation to a Time-Course

The permeation equation to be fitted to an experimental time-course is:

$$-x.f' - (m+x). \ln(1-f') = m.PA.t \quad (2.05)$$

Inspection shows that it is but a special case of the equation that is predicted from the Simple Membrane Carrier Hypothesis, and which is derived in Chapter III, Section 2:

$$-(K+m+x).(K+x).x.f' - (K+x)^2.(m+x). \ln(1-f') = m.KV.t \quad (2.32)$$

Hence equation (2.05) can be fitted by any of the methods developed for equation (2.32).

These methods are described and exhaustively discussed in Chapter III, Section 4. They are based on:

- (1) a direct fit,
- (2) a bivariate first-order fit,
- (3) a trivariate first-order fit.

At least two conclusions emerge from the discussion. They are, firstly, that method (1) is impracticable; and, secondly, that method (2), though of qualified validity, gives more reliable estimates of the characteristic parameters, K and V, than does method (3). For this reason, method (2) was used for fitting both the permeation equations to a time-course. It is elaborated below.

Since equation (2.05) is identical with:

$$\frac{f'}{t} = - \frac{(m+x)}{x} \cdot \frac{\ln(1-f')}{t} - \frac{m.PA}{x}$$

$$\text{or } F = \frac{m+x}{x} \cdot L - \frac{m.PA}{x}$$

$$\text{where } F = \frac{f'}{t} \quad \text{and } L = - \frac{\ln(1-f')}{t}$$

it predicts that the relationship between the variates F and L is of the first order:

$$\text{i.e.} \quad F = b' \cdot L + a'$$

$$\text{where} \quad b' = \frac{m+x}{x}$$

$$\text{and} \quad a' = -\frac{m}{x} \cdot PA \quad (2.23)$$

$$\text{or} \quad L = b'' \cdot F + a''$$

$$\text{where} \quad b'' = \frac{x}{m+x}$$

$$\text{and} \quad a'' = \frac{m}{m+x} \cdot PA \quad (2.24)$$

These predictions are tested thus.

The unweighted first-order regressions of F on L, and of L on F, are calculated by the method of least squares. (Both regressions are required because if the permeation equation does not fit the time-course exactly, they give different, but equally valid, descriptions of the relationship between F and L). Their goodness of fit to the variates is then appraised by inspecting a plot of F against L, and by comparing the residual variance about the regression of F on L with that expected from the anticipated coefficient of variation of  $f'$ . Should the regressions seem to fit the data, the significances of the differences of  $b'$  from  $\frac{m+x}{x}$ , and of  $b''$  from  $\frac{x}{m+x}$ , are indicated by Student's  $t$  - tests. If the differences are not significant, the predictions made from the permeation equation have been fulfilled. The equation is said to fit the time-course, and the best-fit value of PA has meaning. It is equal to the arithmetic mean of the estimates of PA calculated from equations (2.23) and (2.24).

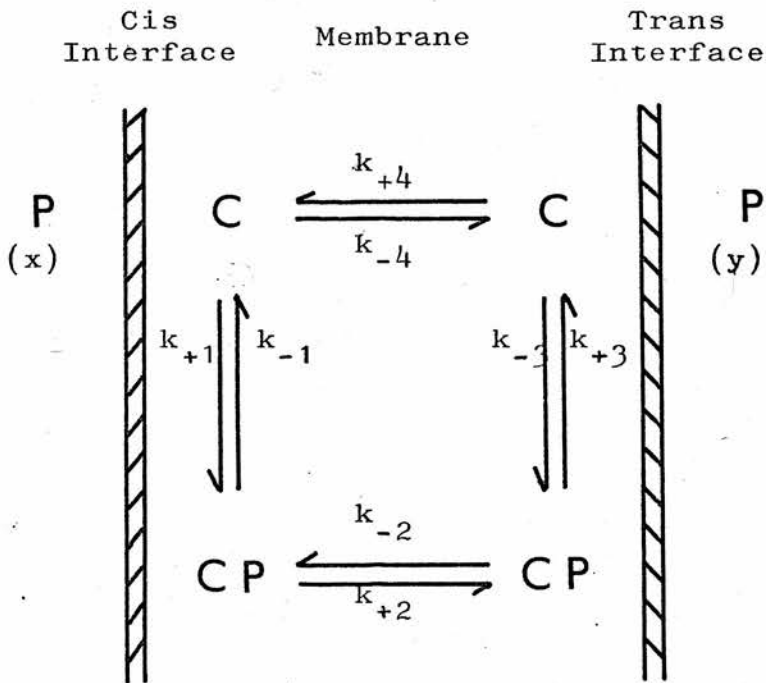
Section 5

Summary

1. The rate of net permeation predicted from the hypothesis is stated (equation (2.01) ).
2. A permeation equation (equation (2.05) ) is derived from the rate equation.
3. Five of the assumptions under-lying the derivation are examined and given qualified support.
4. A method is given for fitting the permeation equation to an observed time-course, and for assessing the goodness of the fit. It is based on the first-order regressions of  $F$  on  $L$ , and  $L$  on  $F$ .

Figure 2.01

A Model of the Simple Membrane Carrier



P represents a molecule of permeant

C represents a carrier

CP represents a carrier-permeant complex .

The osmolalities of the permeant at the cis and trans interfaces are x and y, respectively.

The rate constants for the Simple Membrane Carrier are symmetrical:

$$k_{+1} = k_{-3} = k_1$$

$$k_{-1} = k_{+3} = k_2$$

$$k_{+2} = k_{-4} = k_{-2} = k_{+4} = k_3$$

Widdas (1952) was one of the first to expound the Membrane Carrier Hypothesis which is examined in this Thesis. He proposed that a molecule of permeant crosses the plasma membrane in a manner which is divisible into four reversible stages, and is depicted in Figure 2.01. The stages are:

- (i) The association, at the cis interface, of the permeant, P, with a "carrier", C, which is a specific component of the membrane.
- (ii) The translocation of the carrier-permeant complex, CP, from the cis to the trans interface.
- (iii) The dissociation there of the complex into free carrier and permeant.
- (iv) The translocation of the free carrier back to the cis interface.

In the most general form of the hypothesis, the rate constants for the four stages are those shown in Figure 2.01. However, Widdas suggested, as a simplifying assumption, that the system is symmetrical:

$$\begin{aligned} \text{i.e.} \quad k_{+1} &= k_{-3} = k_1 \\ k_{-1} &= k_{+3} = k_2 \\ k_{+2} &= k_{-2} = k_{+4} = k_{-4} = k_3 \end{aligned}$$

and that  $k_3 < k_2$  (that is, stages (ii) and (iv) are rate-limiting). This symmetrical system is the premise of the Simple Membrane Carrier Hypothesis.



Widdas also derived the kinetics of permeation predicted from the hypothesis. If, at all times, the total number of carriers at the two interfaces,  $N$ , is constant, and if the system is in a steady state, the rate of net flux of permeant,  $\frac{di}{dt}$ , is:

$$\frac{di}{dt} = \frac{N}{2} \cdot \frac{k_1 \cdot k_2 \cdot k_3 \cdot (x - y)}{(k_2 + k_3 + k_1 \cdot x)(k_2 + k_3 + k_1 \cdot y) - k_3^2} \quad (2.25)$$

where  $x$  and  $y$  are the osmolalities of the permeant at the cis and trans interfaces, respectively. This equation can be used to define the rate of net flux of permeant into a cell if the permeant crosses the membrane much less rapidly than does water, and if  $\frac{dx}{dt}$  and  $\frac{dy}{dt}$  are so small that the system is virtually in a steady state. The rate of net flux is expressed more succinctly by letting:

$$\frac{k_2 + k_3}{k_1} = K \quad (2.26)$$

$$\text{and } \frac{N}{2} \cdot \frac{k_2 \cdot k_3}{k_2 + k_3} = w_o \cdot V \quad (2.27)$$

where  $w_o$  is the volume of intracellular water, at isosmolality, of that mass of cells whose membranes have  $N$  carriers. Equation (2.25) then becomes:

$$\frac{di}{dt} = w_o \cdot KV \cdot \frac{(x - y)}{(K+x) \cdot (K+y) - (k_3/k_1)^2} \quad (2.28)$$

Furthermore, since  $k_3 < k_2$ ,  $k_3^2 \ll (k_2 + k_3)^2$ , and equation (2.28) simplifies to:

$$\frac{di}{dt} = w_o \cdot K \cdot V \cdot \frac{(x - y)}{(K+x) \cdot (K+y)} \quad (2.29)$$

This is the equation that defines the kinetics of permeation predicted from the Simple Membrane Carrier Hypothesis.

The characteristic parameters are K and V. They are similar to the half-saturation constant and the maximum velocity, respectively, of an enzyme-catalysed reaction, because equation (2.29) is equivalent to:

$$\frac{di}{dt} = w_o \cdot V \left( \frac{x}{K+x} - \frac{y}{K+y} \right) \quad (2.30)$$

The units of K are those of concentration (e.g. mM). Those of V are: number of molecules transferred per unit volume of intracellular water at isosmolality per unit time; for example: mmol/l intracellular water at isosmolality/min. In most of the present work, this expression is abbreviated to "mM/min".

In view of the apparent difference in the permeability of the human erythrocyte to a- and b- D(+) glucose which is recorded in Part Four, Chapter XV, it is pertinent to consider the kinetics of net permeation of two non-electrolytes, 1 and 2, that share a common carrier. Miller (1965b) has derived expressions which are valid if the two non-electrolytes have different affinity constants for the carrier, but are transferred with the same maximum velocity:

$$\frac{di_1}{dt} = w_o \cdot V \left( \frac{\frac{x_1}{K_1}}{1 + \frac{x_1}{K_1} + \frac{x_2}{K_2}} - \frac{\frac{y_1}{K_1}}{1 + \frac{y_1}{K_1} + \frac{y_2}{K_2}} \right)$$

$$\frac{di_2}{dt} = w_o \cdot V \left( \frac{\frac{x_2}{K_2}}{1 + \frac{x_1}{K_1} + \frac{x_2}{K_2}} - \frac{\frac{y_2}{K_2}}{1 + \frac{y_1}{K_1} + \frac{y_2}{K_2}} \right)$$

The subscripts 1 and 2 refer to the two non-electrolytes, and the symbols, with one exception, have their usual meaning. The exception is "K", with equals  $\frac{k_2}{k_1}$ , and not  $\frac{k_2+k_3}{k_1}$ . Hence these expressions require that  $k_3 \ll k_2$ , and not just that  $k_3^2 \ll (k_2 + k_3)^2$ . The total rate of net permeation,  $\frac{di}{dt}$ , is:

$$\begin{aligned} \frac{di}{dt} &= \frac{di_1}{dt} + \frac{di_2}{dt} \\ &= w_o \cdot V \left\{ \frac{\frac{x_1}{K_1} + \frac{x_2}{K_2}}{1 + \frac{x_1}{K_1} + \frac{x_2}{K_2}} - \frac{\frac{y_1}{K_1} + \frac{y_2}{K_2}}{1 + \frac{y_1}{K_1} + \frac{y_2}{K_2}} \right\} \end{aligned}$$

If, at any instant,  $x_1 + x_2 = x$ ,  $x_1 = u \cdot x$ ,  $x_2 = v \cdot x$ ,  $y_1 + y_2 = y$ ,  $y_1 = u \cdot y$  and  $y_2 = v \cdot y$ , where  $u$  and  $v$  are constants:

$$\frac{di}{dt} = w_o \cdot V \left\{ \frac{x}{K_o + x} - \frac{y}{K_o + y} \right\} \quad (2.31)$$

$$\text{where } K_o = \frac{K_1 \cdot K_2}{v \cdot K_1 + u \cdot K_2}$$

Equation (2.31) is identical with equation (2.30), except that  $K_o$  and  $K$  are defined in different ways.

This Chapter is divided into five Sections, which are similar to those of its predecessor. A permeation equation is derived (Section 2), and two of the assumptions on which it is founded are discussed (Section 3).

In Section 4, three methods for fitting the permeation equation to an experimental time-course are considered, and one is chosen. Finally, Section 5 is a Summary.

## Section 2 The Permeation Equation

The permeation equation is derived by integrating with appropriate boundary conditions the expression that defines the rate of net permeation of solute into a mass of cells. The simplest form of the expression is:

$$\frac{di}{dt} = w_o \cdot K \cdot V \cdot \frac{(x - y)}{(K+x) \cdot (K+y)} \quad (2.29)$$

A typical experiment is identical with the one described in Chapter II, Section 2. To recapitulate: the mass of cells is in osmotic equilibrium with an isosmolal suspension medium of osmolality  $m$ . At time zero, permeant is added to this medium and the total extracellular osmolality rises to  $(m+x)$ . At time  $t$ ,  $i$  osmoles of permeant have entered the cells. If the system fulfils the four provisos listed on p.28, the volume of intracellular water at this instant,  $w$ , is:

$$w = \frac{m \cdot w_o + i}{m+x} \quad (2.02)$$

$$\text{and hence } y = \frac{(m+x) \cdot i}{m \cdot w_o + i} \quad (2.03)$$

Equation (2.29) can now be integrated, provided that  $K$  and  $V$  are constants. If the boundary condition is that  $i = 0$  when  $t = 0$ , and if  $i/(x \cdot w_o) = f'$ , the integration gives:

$$-(K+m+x) \cdot (K+x) \cdot x \cdot f' - (K+x)^2 \cdot (m+x) \cdot \ln(1-f') = m \cdot K \cdot V \cdot t \quad (2.32)$$



### Section 3 The Applicability of the Permeation Equation to the Human Erythrocyte

The argument in Chapter II, Section 3, indicates that the permeation equation is only an approximation. There are two main reasons for this. Firstly, because the number of osmoles of intracellular material varies, the equation giving  $w$ , the volume of intracellular water, is not equation (2.02), but:

$$w = \frac{((m - 0.0395) \cdot w_o + i) + (((m - 0.0395) \cdot w_o + i)^2 + 0.158 \cdot (m + x) \cdot w_o^2)^{\frac{1}{2}}}{2(m + x)} \quad (2.20)$$

And secondly, the osmolality of the extracellular medium, and of the permeant in it, is not constant, but changes with time. The accuracy of the approximation was investigated in a manner exemplified thus.

Although equation (2.29) cannot be integrated algebraically when  $w$  is given by equation (2.20) rather than by equation (2.02), it can be integrated numerically. Therefore  $K$ ,  $V$ ,  $m$ ,  $x$  and  $w_o$  were given specific, or "true", values, and the theoretical time-course was computed. The permeation equation, 2.33, was then fitted to the time-course, and apparent values of  $K$  and  $V$  were calculated from the fit. The difference between the true and the apparent values of  $K$  and  $V$  is a measure of the accuracy of the approximation.

The investigation was conducted in two independent stages. In the first, the extracellular osmolality was assumed to be constant, and  $w$  was calculated from equation (2.20). Then, with the number of osmoles of intracellular





Table 2.01

The Effect of the Variation of the Number of Osmoles of Intracellular Material on the Apparent Values of K and V

Time-course	x	$10^4 \cdot c(b')$	$K' \pm \text{S.E.}$	$V' \pm \text{S.E.}$
(a)	320	4.4	$78.1 \pm 0.4$	$114.8 \pm 0.6$
(b)	75	1.8	$78.3 \pm 0.0$	$112.6 \pm 0.1$

$K'$ ,  $V'$  are the apparent values of  $K, V$  respectively.

$c(b')$  is the coefficient of variation of  $b'$   
 $(F = b' \cdot L + a')$ .

The true values of  $K$  and  $V$  are 75 and 110, respectively.

material assumed constant, the effect of the variation in extracellular osmolality was studied. The stages are set out below.

### 3.01 The Variation of the Number of Osmoles of Intracellular Material

Equation (2.29), with  $w$  being given by equation (2.20), was integrated numerically by the Kutta-Merson process (Mayers, 1962). The computations were executed in the University of Edinburgh's KDF 9 Computer (English Electric Co., Ltd.)

Two typical time-courses, each of 8 points ( $0.23 \leq f' \leq 0.78$ ), were constructed. They are supposed to represent the permeation of L(+) arabinose (see Part Four, Chapter XV). Hence  $K$  and  $V$  were set to 75 and 110, respectively, and the osmolality of extracellular non-electrolyte was chosen to be (a) high ( $m = 300$ ,  $x = 320$ ); and (b) low ( $m = 300$ ,  $x = 75$ ). The permeation equation, 2.33, was fitted to each time-course by the method based on two bivariate first-order regressions (see Section 4.02 of this Chapter). The results are summarised in Table 2.01. In both instances, a plot of  $F$  against  $L$  was concave (i.e.  $d^2F/dL^2$  was positive), but scarcely departed from linearity. Nevertheless, although the differences between the true and apparent values of  $K$  and  $V$  are small, they are real.

Table 2.02

The Effect of the Variation of the Extracellular Osmolality  
on the Apparent Values of K and V

Time-course	W	x	mean	range	$10^4 \cdot c(b')$	$K' \pm \text{S.E.}$	$V' \pm \text{S.E.}$
(c)	10		313.0	314.3-311.7	2.4	$75.8 \pm 0.2$	$110.3 \pm 0.3$
(d)	5		306.4	308.9-303.8	5.6	$76.4 \pm 0.4$	$110.7 \pm 0.7$
(e)	10		73.4	74.1-72.9	69.1	$70.2 \pm 1.3$	$109.1 \pm 2.5$
(f)	5		71.9	73.2-71.0	140.0	$65.2 \pm 2.4$	$108.1 \pm 5.1$

$K'$ ,  $V'$  are the apparent values of K, V respectively.

$c(b')$  is the coefficient of variation of  $b'$  ( $F=b' \cdot L+a'$ ).

W is the initial volume of the extracellular medium.

For each time-course,  $\bar{m} = 300$ ,  $w_0 = 0.3$ ,  $K = 75$ ,  $V = 110$ .

### 3.02 The Variation of the Osmolality of the Extracellular Medium

Equation (2.29) was again integrated numerically by the Kutta-Merson process. On this occasion,  $w$  and  $x$  were calculated from equations (2.21) and (2.22), respectively. Four typical  $L(+)$  arabinose time-courses, (c) through (f), were computed. Table 2.02 shows the values allotted to the constants, the mean and range of  $x$ , and the apparent values of  $K$  and  $V$ . These apparent values were found from the coefficients of the two bivariate first-order regressions, and the mean of  $x$ . In every instance, a plot of  $F$  against  $L$  was concave. It is interesting that  $K$  and  $V$  were overestimated when  $x = 320$ , but underestimated when  $x = 75$ . This emphasises that the estimates depend on the number and magnitudes of the data comprising the time-course. It also seems that the accuracy of the estimates decreases as  $\frac{x}{K}$  decreases, especially when  $W$ , the initial volume of the extracellular medium, has its lower value of 5.

### 3.03 Discussion

The estimates of  $K$ , derived by fitting the permeation equation to a typical  $L(+)$  arabinose time-course, are less accurate than those of  $V$ . However, all these systematic deviations are small compared with the random deviations in  $K$  and  $V$  caused by experimental error (see Section 4.04 of this Chapter). There is no reason to suppose that the latter conclusion does not apply to permeants for which  $K$



and  $V$  are not respectively 75 and 110. Therefore little is gained by modifying the permeation equation - provided that  $w_0$ , the initial volume of intracellular water, is small compared with  $W$ . Since, in the experiments described in Part Three, the ratio  $w_0:W$  was always less than 0.3:5, no modification is needed.

#### Section 4 Fitting the Permeation Equation to a Time-Course

The permeation equation to be fitted to an experimental time-course is:

$$-(K+m+x).(K+x).x.f' - (K+x)^2.(m+x). \ln(1-f') = m.K.V.t \quad (2.32)$$

Three methods were considered for estimating the best-fit values of K and V, and for appraising the goodness of the fit, given that m, x and t are known precisely, but that f' may contain experimental error.

They are based on:

- (1) a direct fit;
- (2) a bivariate first-order fit;
- (3) a trivariate first-order fit.

They are elaborated and discussed in the rest of this Section.

##### 4.01 The Direct Fit Method

Equation (2.32) is equivalent to:

$$\bar{A}.f' + \bar{B}.\ln(1-f') = t \quad (2.33)$$

K and V are found from  $(\bar{A}_{\min}, \bar{B}_{\min})$ , which are the values of  $\bar{A}$  and  $\bar{B}$  that minimise the sum of the squares of the differences between the values of f' actually observed and those computed from equation (2.33).

For example, suppose that an experimental time-course has n points  $(t_i, f_i')$ , and that the variance of  $f_i'$  is  $(1/w_i)$ . Initial estimates of  $\bar{A}$  and  $\bar{B}$ ,  $(\bar{A}_1, \bar{B}_1)$  are chosen. The value of f',  $F_i'$ , that is predicted from equation (2.33) to correspond to  $t_i$ , is computed by solving the equation numerically. The quantity  $\bar{S}_{11}$ , which is equal to

$\sum_{i=1}^n w_i \cdot (f_i' - F_i')^2$ , is summed. The whole computation is repeated with  $(pq - 1)$  different pairs of values of  $\bar{A}$  and  $\bar{B}$  ( $\bar{A}_j, \bar{B}_k$ ; where  $1 \leq j \leq p$ , and  $1 \leq k \leq q$ ), so that the relationship of  $\bar{S}$  to  $\bar{A}$  and  $\bar{B}$  is "mapped". If  $\bar{S}$  has a minimum within this area, its co-ordinates ( $\bar{A}_{\min}, \bar{B}_{\min}$ ), and value ( $\bar{S}_{\min}$ ), are determined.

A grid of nine points ( $\bar{A}, \bar{B}, \bar{S}$ ) that bounds the minimum is selected, and a general second-order equation,  $\bar{S} = g(\bar{A}, \bar{B})$ , is fitted to them. The values of  $\bar{A}$  and  $\bar{B}$  for which this equation has a minimum are first estimates of ( $\bar{A}_{\min}, \bar{B}_{\min}$ ); they are found by solving for  $\bar{A}$  and  $\bar{B}$  the simultaneous equations  $\frac{d\bar{S}}{d\bar{A}} = 0$  and  $\frac{d\bar{S}}{d\bar{B}} = 0$ . The procedure is repeated with successively smaller grids of nine points until the estimates of ( $\bar{A}_{\min}, \bar{B}_{\min}$ ) and  $\bar{S}_{\min}$  are sufficiently accurate.

The best-fit values of  $K$  and  $V$  are derived from  $\bar{A}_{\min}$  and  $\bar{B}_{\min}$ . Since the variances and covariance of  $\bar{A}_{\min}$  and  $\bar{B}_{\min}$  are known functions of the coefficients of  $\bar{S}_{\min} = g(\bar{A}_{\min}, \bar{B}_{\min})$ , approximate variances of the best-fit values are available.

The goodness of the fit of the equation to the time-course is indicated by the magnitude of the residual variance,  $\frac{1}{n-2} \cdot \bar{S}_{\min}$ , and by the form of the distribution of  $(f_i' - F_i')$  with  $t_i$ . If the residual variance is accounted for by experimental error, and if the distribution seems to be random, the equation is said to fit the time-course.

The principal merit of the method is that it minimises  $\sum (f_i' - F_i')^2$ , and gives approximate variances, as well as the actual magnitudes, of the carrier parameters. Unfortunately, preliminary investigations using simulated data suggested that the method would be not only expensive in computer time, but also unreliable, in that small errors in the data would cause successive estimates of  $\bar{A}_{\min}$  and  $\bar{B}_{\min}$  to oscillate rather than converge. They also showed that if  $f'$  did not exceed about 0.4, the coefficient of correlation between  $\bar{A}_{\min}$  and  $\bar{B}_{\min}$  was nearly unity. This is because, when  $f'$  is small, it is directly proportional to  $-\ln(1-f')$ . In consequence, accurate estimates of  $K$  and  $V$  can only be obtained from time-courses which have values of  $f'$  greater than 0.4.

The method could have been refined by the use of other techniques for optimising  $\bar{A}$  and  $\bar{B}$ , such as those described by Swann (1969). But because the development of a suitable computer program would have been expensive, the two first-order methods for fitting the permeation equation were examined.

#### 4.02 The Bivariate First-Order Fit Method

Equation (2.32) can be re-arranged to:

$$\frac{f'}{t} = - \frac{(K+x) \cdot (m+x)}{(K+m+x) \cdot x} \cdot \frac{\ln(1-f')}{t} - \frac{m \cdot K \cdot V}{(K+m+x) \cdot (K+x) \cdot x}$$



or, if  $F = \frac{f'}{t}$  and  $L = - \frac{\ln(1-f')}{t}$ , to:

$$F = \frac{(K+x) \cdot (m+x)}{(K+m+x) \cdot x} \cdot L - \frac{m \cdot K \cdot V}{(K+m+x) \cdot (K+x) \cdot \bar{x}} \quad (2.34)$$

The goodness of fit of equation (2.34) to a time-course is indicated by the order of, and the residual variance about, the polynomial relating F and L. If the order does not differ significantly from unity, and if the residual variance about the first-order polynomial can be attributed to experimental error, equation (2.34) is said to fit the time-course. The Best-fit values of K and V, and their variances, are meaningful and can be calculated from the coefficients of this polynomial, and the residual variance about it.

Because F and L are both functions of t and f', the polynomial is not identical with the least-squares regression of either (a), F on L, or (b), L on F. If this point is ignored, there is no a priori reason for representing the polynomial with one, rather than the other, of the two regressions. Furthermore, for neither of the regressions is the "variance" of an individual value of the "dependent" variate clearly defined.

If the first-order regression of F on L, of coefficients b' and a', is identified with the polynomial, then:

$$b' = \frac{(K+x) \cdot (m+x)}{(K+m+x) \cdot x}$$

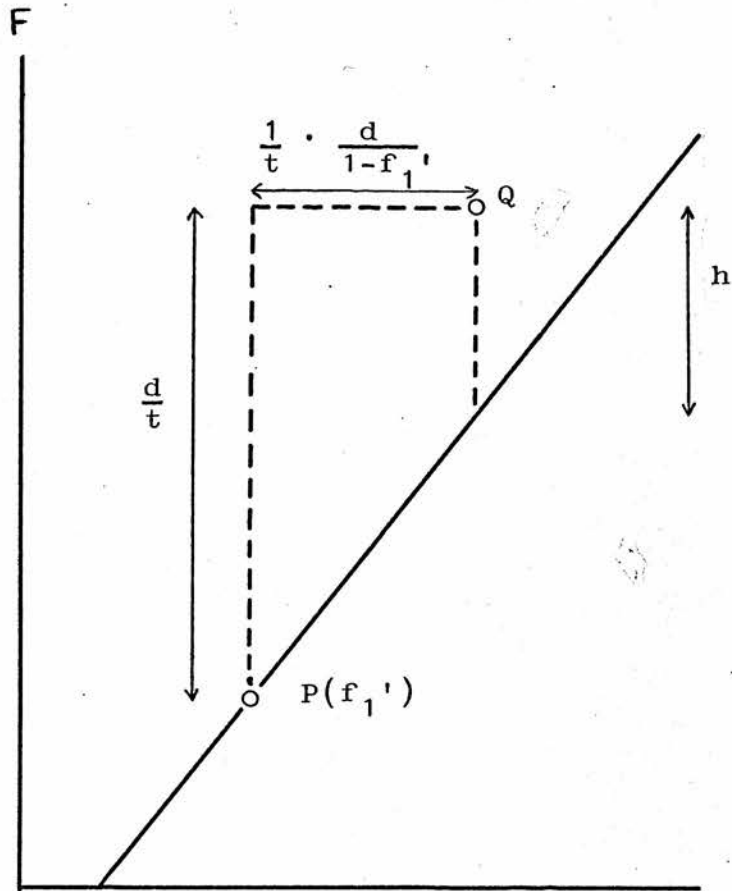
$$\text{and } a' = - \frac{m \cdot K \cdot V}{(K+m+x) \cdot (K+x) \cdot x}$$

$$\text{so that } K' = (b' - 1) \cdot \frac{x \cdot (m+x)}{m - (b' - 1) \cdot x}$$

$$\text{and } V' = - a' \cdot \frac{(K+m+x) \cdot (K+x) \cdot x}{K \cdot m}$$

Figure 2.02

The Effect of an Error in  $f'$  on  
a Plot of  $F$  against  $L$



$$F = f'/t; \quad L = \frac{-\ln(1-f')}{t}$$

$$F = b' \cdot L + a'$$

The error in  $f_1'$  is  $d$ : it causes the point  $P$  to be transposed to  $Q$ .

where  $K'$  and  $V'$  are the best-fit estimates of  $K$  and  $V$ .

On the other hand, if the first-order regression of  $L$  on  $F$ , of coefficients  $b''$  and  $a''$ , is identified with the polynomial:

$$K'' = (1-b'') \cdot \frac{x(m+x)}{m \cdot b'' - (1-b'') \cdot x}$$

$$\text{and } V'' = \frac{a''}{b''} \cdot \frac{(K+m+x) \cdot (K+x) \cdot x}{K \cdot m}$$

where  $K''$  and  $V''$  are the best-fit estimates of  $K$  and  $V$ .

When the residual variances about the regressions are not zero, the product of  $b'$  and  $b''$  is not unity, and the regressions lead to different estimates of the carrier parameters. How these estimates should be combined is not obvious.

In both regressions, the uncertainty in the variance of the "dependent" variate arises because the experimentally-determined quantities,  $t$  and  $f'$ , have not been separated. For example, the variance of  $F$  is not  $(1/t^2) \cdot \text{var}(f')$ . This can be shown as follows. Suppose that, in Figure 2.02, the point  $P(f_1')$  obeys equation (2.34). If  $f_1'$  is changed, by experimental error, to  $(f_1' + d)$ ,  $F(=(f_1')/t)$  becomes  $(f_1' + d)/t$ , and  $L(= -(1/t) \cdot \ln(1-f_1'))$  becomes  $-(1/t) \cdot \ln(1-f_1' - d)$ . The last quantity can be expanded in an infinite Taylor series:

$$\begin{aligned} -\frac{\ln(1-f_1' - d)}{t} &= -\left\{ \frac{\ln(1-f_1')}{t} + \frac{1}{t} \cdot \frac{d}{1-f_1'} + \frac{1}{2t} \cdot \left( \frac{d}{1-f_1'} \right)^2 + \dots \right\} \\ &\approx -\left\{ \frac{\ln(1-f_1')}{t} + \frac{1}{t} \cdot \frac{d}{1-f_1'} \right\} \end{aligned}$$

when  $d \ll (1-f_1')$ .

Therefore the point  $P$ , originally on the straight line defined by the equation, is transposed to  $Q$ , which lies a distance  $h$ , parallel to the ordinate, from the line, where

$$h = (d/t) \cdot (1-b'/(1-f_1'))$$

Hence the effective variance of  $F$  may be:

$$(1-b'/(1-f'))^2 \cdot (1/t)^2 \cdot \text{var}(f')$$

where  $f'$  is the true or, to a first approximation, the observed value of  $f'$ .

In consequence, there are two difficulties about using the regression of  $F$  on  $L$ . The first is that the factor by which an individual value of  $F$  ought to be weighted may be the reciprocal of its effective variance, rather than the reciprocal of  $(1/t^2) \cdot \text{var}(f')$ .

The second difficulty is that the magnitude of the residual variance about a regression may have limited meaning. It follows that the significance of the difference from unity of the order of a multi-order regression cannot be ascertained by comparing the variance about the first-order regression with that removed by the addition of the higher-order terms; that the residual variance about the first-order regression may not be comparable to that expected from experimental error; and that the standard errors of  $K'$  and  $V'$ , derived from those of  $b'$  and  $a'$ , may not be reliable.

The same difficulties apply to the use of the regression of  $L$  on  $F$ . Thus, before a bivariate first-order fit can be used to find  $K$  and  $V$ , five problems must be solved. They are those of:

- (i) Weighting correctly an individual value of a dependent variate.
- (ii) Ascertaining the order of the best-fit regression relating  $F$  and  $L$ .
- (iii) Estimating the magnitude of the residual variance about a first-order regression that can be attributed to a known experimental error.
- (iv) Averaging correctly the estimates of  $K$  and  $V$  (that is:  $K', K'', V', V''$ ) derived from

the two first-order regressions.

- (v) Assessing the significance of the standard errors of K and V that are derived from the standard errors of the coefficients of a first-order regression.

The second problem can be solved subjectively by inspecting a plot of F against L, and classifying its departure from linearity in a manner comparable to that proposed by Matthews (1966). The other problems were partly solved by analysing simulated time-courses. This analysis, which is also relevant to the method based on a trivariate first-order fit, is deferred to Section 4.04.

#### 4.03 The Trivariate First-Order Fit Method

This method is similar to that based on a bivariate first-order fit. Since the permeation equation is:

$$-(K+m+x).(K+x).x. f' - (K+x)^2.(m+x).ln(1-f') = m.K.V.t \quad (2.32)$$

its goodness of fit to a time-course is indicated by the order of the polynomial, with no constant term, relating  $f'$ ,  $ln(1-f')$ , and  $t$ , and by the residual variance about it.

For the reasons mentioned in the preceding sub-section, the polynomial is not identical with any of the three possible least-squares regressions:

- i.e. those of (c)  $t$  on  $f'$  and  $-ln(1-f')$
- (d)  $f'$  on  $-ln(1-f')$  and  $t$
- (e)  $-ln(1-f')$  on  $t$  and  $f'$ .

If the regressions are used when the polynomial does not fit the time-course perfectly, they yield different estimates of K and V. Moreover, it is not apparent, either



by what factor an individual value of a dependent variate ought to be weighted, or what significance can be attached to the residual variance about a regression. In short, the proposed use of this method raises problems which are analogous to those posed by the examination of the method based on a bivariate first-order fit.

These problems were also partly solved by analysing simulated time-courses. The analysis is described below.

#### 4.04 An Analysis of Simulated Time-Courses

The analysis was performed in an attempt to solve four of the problems which were raised by the examination of the two first-order methods for finding the best fit of the permeation equation to a time-course. It was carried out in four stages.

The first was the simulation of time-courses which would, but for their content of "experimental error", be defined by the permeation equation. Secondly,  $K$  and  $V$ , and their standard errors, were estimated from these time-courses, using all five unweighted first-order regressions. To recapitulate: regressions (a) and (b) are those of  $F$  on  $L$ , and of  $L$  on  $F$ , respectively (see p. 58), while regressions (c), (d) and (e) are introduced in Section 4.03. The estimates were compared with the "true" values of the parameters - that is, the numerical values substituted for  $K$  and  $V$  when the time-courses were simulated. Thirdly,  $K$  and  $V$  were re-calculated, using a weighted form of regression (a). Finally, the residual variances about

both the unweighted and the weighted forms of regressions (a) were compared with that predicted from the known variability of the time-courses.

The simulation of an experimental time-course can be summarised as follows. A "perfect", or error-free, time-course is formed by substituting the constants  $m$ ,  $x$ ,  $K$  and  $V$  in equation (2.32) with suitable numerical values, and calculating  $t$  at chosen values of  $f'$ . Error is introduced into the perfect time-course by multiplying each value of  $f'$ , the variable subject to error, with a different pseudo-random number. The pseudo-random numbers are members of an infinite series whose mean is unity and whose standard deviation is equal to the coefficient of variation expected for that value of  $f'$ . A simulated experimental time-course, of known variability, comprises the original values of  $t$  and the randomised values of  $f'$  corresponding to them.

In the experiments with monosaccharides, described in Part Four, the concentrations of extracellular permeant ranged from 320 mM (L(+) arabinose) to 20 mM (D(+) glucose), the apparent values of  $K$  lay between about 75 mM (L(+) arabinose) and 5 mM (D(+) glucose), and the apparent values of  $V$  were all roughly 110 mM/min. Two perfect time-courses, determined in extreme conditions, were therefore formed. The first represents the permeation of L(+) arabinose when the extracellular concentration is high; the second, that of D(+) glucose when the extracellular concentration is low.

The perfect L(+) arabinose time-course was formed by

Table 2.03

The Theoretical Relationship between  $f'$  and its Coefficient  
of Variation, for Arabinose

$f'$	0.2	0.3	0.4	0.5	0.66	0.7	0.8	0.9
$c(f')(\%)$	1.46	1.00	0.78	0.64	0.55	0.48	0.43	0.40

The coefficient of variation of  $f'$  is  $c(f')$ .

. The calculation of the relationship is outlined on p.109 .

setting  $m, x, K$  and  $V$  to 300, 320, 75 and 110, respectively, and calculating  $t$  at eight approximately equispaced values of  $f'$  between 0.23 and 0.77. Although the coefficient of variation of  $f'$  was never measured accurately, its theoretical value can be derived from the coefficients of variation of the methods for estimating pentose and inulin, and the ratio of the volume of water within the erythrocytes to the volume of water in the lower compartment of a waisted centrifuge tube (see p.109 ). These minimum values, in a typical time-course, are shown in Table 2.03. Since they are probably less than the values realised in practice (see p.152), "high-error" as well as "low-error" time-courses were simulated. For the latter, the coefficient of variation chosen for  $f'$  was 1.0% when  $0.23 \leq f' \leq 0.37$ , and 0.5% when  $0.45 \leq f' \leq 0.77$ . For the former, it was increased some five-fold, to 5.0% when  $0.23 \leq f' \leq 0.45$ , and 2.5% when  $0.54 \leq f' \leq 0.77$ .

Four series of pseudo-random numbers, with means of 1.000 and standard deviations of 0.005, 0.010, 0.025, and 0.050, respectively, were generated in the University of Edinburgh's KDF 9 Computer. The program for "Random K", the sub-routine used to generate the numbers, was obtained from the University Computer Unit's program library. Ten different "low-error" time-courses were formed from the perfect time-course and the first two series of numbers, and ten different "high-error" time-courses were formed from the same perfect time-course and the second two series of numbers.

Table 2.05

The Values of K and V from the Simulated "High-Error"  
L(+) Arabinose Time-Courses

Experiment	(a)			(b)			(c)			(d)			(e)		
	K	V		K	V		K	V		K	V		K	V	
1	95.0 $\pm$ 15.1	103.9 $\pm$ 18.0		96.8	91.6		164.0	88.6		480.8	93.3		771.2	113.7	
2	82.0 $\pm$ 27.6	102.2 $\pm$ 37.0		88.1	99.4		303.0	83.0		260.7	82.2		283.1	82.5	
3	90.7 $\pm$ 19.3	96.9 $\pm$ 22.5		93.6	95.8		101.6	93.6		80.7	103.7		90.2	98.4	
4	76.3 $\pm$ 21.8	108.5 $\pm$ 33.2		80.2	106.3		128.3	89.6		82.5	106.8		103.2	96.6	
5	73.6 $\pm$ 27.9	107.3 $\pm$ 43.3		80.0	103.5		89.1	99.5		68.7	113.3		777.8	106.1	
6	64.8 $\pm$ 22.7	115.1 $\pm$ 42.5		69.1	111.6		27.9	204.9		-	-		-	-	
7	40.5 $\pm$ 26.3	150.3 $\pm$ 100.2		46.4	138.0		182.2	81.5		134.3	86.9		157.1	83.6	
8	30.5 $\pm$ 23.8	191.8 $\pm$ 151.7		35.5	172.6		80.5	104.8		50.7	136.5		63.5	118.7	
9	86.3 $\pm$ 31.0	106.9 $\pm$ 41.4		94.1	103.5		78.0	112.9		58.5	132.5		67.3	122.1	
10	58.7 $\pm$ 9.5	119.4 $\pm$ 20.4		59.4	118.6		54.7	124.9		48.7	134.5		51.3	130.1	
Mean	69.8	120.2		74.3	114.1		120.9	108.3		140.6	110.0		185.0	105.7	
S.D.	21.3	29.2		21.2	24.4		79.4	36.6		143.9	20.8		231.2	16.8	
S.E.M.	6.7	9.2		6.7	7.7		25.1	11.6		48.0	6.9		77.1	5.6	

K and V were calculated using the first-order regressions  
(a) through (e), which are defined in Section 4.04.  
The "true" values of K and V are 75 and 110, respectively.



Table 2.04

The Values of K and V from the Simulated "Low-Error"

L(+) Arabinose Time-Courses

Experiment	(a)			(b)			(c)			(d)			(e)		
	K			V	K	V	K	V		K	V		K	V	
1	71.5 $\pm$ 4.3	112.7 $\pm$ 7.2			71.7	112.6	83.1	104.8		82.5	105.1		82.8	105.0	
2	85.3 $\pm$ 4.1	104.1 $\pm$ 5.4			85.4	104.0	73.2	111.9		72.4	112.5		72.7	112.2	
3	70.0 $\pm$ 5.4	114.1 $\pm$ 9.3			70.2	114.0	102.2	996.4		98.0	98.0		99.9	97.2	
4	72.2 $\pm$ 6.8	112.2 $\pm$ 11.3			72.6	112.0	68.5	115.5		67.6	116.4		68.0	116.0	
5	64.4 $\pm$ 1.7	117.7 $\pm$ 3.3			64.4	117.7	57.4	125.5		57.2	125.8		57.3	125.7	
6	77.3 $\pm$ 5.4	108.6 $\pm$ 8.1			77.6	108.4	87.7	102.3		84.1	104.3		85.8	103.4	
7	79.3 $\pm$ 5.4	107.7 $\pm$ 7.9			79.6	107.6	114.8	92.9		110.6	94.2		112.6	93.6	
8	73.0 $\pm$ 2.8	117.7 $\pm$ 3.7			73.0	111.7	83.2	104.9		82.9	105.1		83.0	105.0	
9	79.1 $\pm$ 5.8	110.0 $\pm$ 8.7			79.4	109.8	93.7	102.0		91.4	103.1		92.5	102.5	
10	78.5 $\pm$ 3.3	107.9 $\pm$ 4.8			78.6	107.9	74.2	110.9		73.7	111.3		76.8	108.8	
Mean	75.1	110.7			75.2	110.6	83.8	106.7		82.0	107.6		83.1	106.9	
S.D.	6.0	3.9			6.0	3.9	16.8	9.6		15.5	9.2		16.0	9.3	
S.E.M.	1.9	1.3			1.9	1.2	5.3	3.1		4.9	2.9		5.1	2.9	

K and V were calculated using the first-order regressions

(a) through (e), which are defined in Section 4.04.

The "true" values of K and V are 75 and 110, respectively.

Tables 2.04 and 2.05 show the five pairs of estimates of K and V which were calculated for each of the twenty time-courses from the coefficients of the five unweighted first-order regressions. Also shown are the apparent standard errors of K and V derived from the standard errors of  $b'$  and  $a'$ , the coefficients of regression (a). It may be observed that:

- (i) The Estimates of K and V from regressions (a) and (b) are nearly equal, and the same is true of those from regressions (c), (d) and (e). But the former estimates are both more accurate and more precise than the latter. This suggests that in neither the bivariate nor the trivariate regressions did the relationship between the variates depart much from linearity: a suggestion supported by the small coefficients of variation of  $b'$  in the "low-error" and the "high-error" time-courses (0.55% and 2.78%, respectively). However, it is also evident that a variation in  $f'$  alters the "dependent" variate of a bivariate regression in a manner whose dependence on  $f'$  is different from that of the equivalent variation in the "dependent" variate of a trivariate regression.
- (ii) For any time-course, V is in general underestimated when K is overestimated; and vice versa. The product KV tends to be more accurate than are either K or V alone.
- (iii) For both the "low-error" and the "high-error" time-courses, the "apparent" standard error of each estimate of K agrees quite closely with the "true" standard deviation of K (that is, the standard deviation of the ten estimates of K). This suggests that the standard

error calculated for  $b'$  is quantitatively meaningful. On the other hand,  $V$  has an apparent standard error which is, on average, greater than its "true" standard deviation. This may be due to the omission of the covariance of  $b'$  and  $a'$  from the expression for the former quantity.

- (iv) All five regressions gave estimates of  $V$  which are more precise than those of  $K$ . But the coefficients of variation of both  $K$  and  $V$ ,  $c(K)$  and  $c(V)$  respectively, are much greater than the coefficient of variation of  $f'$ ,  $c(f')$ . For example, for the estimates calculated from regression (a):

"low-error" time-courses:

$$c(f') = 0.5 - 1.0\%; \quad c(K) = 8.0\%; \quad c(V) = 3.5\%$$

"high-error" time-courses:

$$c(f') = 2.5 - 5.0\%; \quad c(K) = 30.5\%; \quad c(V) = 24.3\%$$

In the third stage of the analysis, an attempt was made to improve the reliability of the estimates of  $b'$  and  $a'$ , and hence of  $K$  and  $V$ , by weighting the individual points in a regression of  $F$  on  $L$ . Two weighting functions were used. The first was derived on the assumption that the variance of  $F$  is  $\left(\frac{f' \cdot c(f')}{t}\right)^2$ , and is the reciprocal of this variance. The second is the reciprocal of the quantity that may be the effective variance of  $F$ : i.e.  $\left(\frac{f' \cdot c(f') \cdot (1-b'/(1-f'))}{t}\right)^{-2}$  (see p. 59).

The weight given to an individual point was found from a weighting function by equating  $c(f')$  to its known value, and  $f'$ ,  $t$  and  $b'$  to their "perfect" values.  $K$  and  $V$  were re-estimated from the ten "low-error" time-courses, using

the first weighting function. The means and standard deviations are:

$$K = 79.6 \pm 13.6 \quad V = 108.8 \pm 8.5$$

These standard deviations are significantly greater than those of the values of K and V calculated from the unweighted regression of F on L (variance ratio test;  $P < 0.05$ ).

The second weighting function was used in the recalculation of K and V from both the "low-error" and the "high-error" time-courses. The means and standard deviations are:

$$\text{"low-error" data: } K = 73.9 \pm 6.0 \quad V = 111.4 \pm 3.8$$

$$\text{"high-error" data: } K = 73.1 \pm 21.3 \quad V = 117.8 \pm 26.4$$

Since they do not differ much from those calculated from an unweighted regression of F on L, it seems that the reliability of the estimates of K and V is not improved by using this weighting function.

The second "perfect" time-course represents the permeation of D(+) glucose, and is similar to one determined experimentally (see Table 4.09, facing p.154). The constants m, x, K and V in equation (2.32) were given numerical values of 300, 35, 5 and 50 respectively, and seven equispaced values of  $f'$  were chosen, within the range 0.39 to 0.75. It is shown in Table 4.02 (facing p. 148) that when the permeant is glucose, a realistic value of the coefficient of variation of  $f'$  is 4% for all values of  $f'$ .

Ten "experimental" time-courses were simulated and

Table 2.06

The Values of K and V from the Simulated  
D(+) Glucose Time-Courses

Regression	K $\pm$ S.D.	V $\pm$ S.D.
(a)	5.37 $\pm$ 3.44	62.0 $\pm$ 23.2
(b)	6.84 $\pm$ 3.83	50.7 $\pm$ 15.2
(c)	12.84 $\pm$ 6.95	42.5 $\pm$ 18.2
(f)	5.19 $\pm$ 3.33	63.0 $\pm$ 23.8

Each value of K and V is the mean of 10 values.

The individual values were calculated using the first-order regressions (a), (b), (c) and (f), which are defined in Section 4.04.

The "true" values of K and V are 5 and 50, respectively.



analysed by methods qualitatively identical with those used for the "L(+) arabinose" data. Since the results are qualitatively similar to those for L(+) arabinose, they are not presented in detail, but are summarised in Table 2.06. Regressions (a), (b) and (c) were defined on p. 62, and (f) is the weighted first-order regression of F on L when the weighting function is

$$\left( \frac{f' \cdot c(f') \cdot (1-b'/(1-f'))}{t} \right)^{-2} .$$

Student's t-tests showed that all three bivariate regressions (that is, (a), (b) and (f)), gave estimates of K and V which do not differ significantly from the true values of 5 and 50 respectively ( $P > 0.05$ ), whereas the trivariate regression, (c), yielded an estimate of K which is significantly different from the true value ( $P < 0.001$ ). Variance ratio tests showed that none of the four standard deviations of V differ from one another ( $P > 0.05$ ), but that the standard deviation of K derived from the trivariate regression is significantly greater than those derived from the three bivariate regressions ( $P < 0.05$ ). Therefore the bivariate regressions give estimates of K and V which are not biased, and which are more reliable than those given by the trivariate regressions. Nevertheless, even the minimum values of the coefficients of variation of K and V (64% and 38% respectively) are large when compared with the coefficient of variation of  $f'$  (4%).

It is evident that, strictly, any conclusions drawn from the simulated time-courses are valid only for these

time-courses, because the conclusions are determined by the values of  $K$ ,  $V$ ,  $m$  and  $x$ , as well as by the number, the co-ordinates and the variability of the points describing each time-course. However, the two "perfect" time-courses are representative of those found in two extreme sets of conditions: namely, when  $x$  and  $K$  are both large, and when they are both small. Furthermore, the number, the distribution and the variability of the individual points in each of the simulated "experimental" time-courses approximate to those of practice. Thus it is reasonable to assume that the conclusions are valid for all the time-courses which have been determined experimentally.

In consequence, the solutions to three of the problems ( (i), (iv) and (v) ) first posed on p.60 are that:

- (i) an individual value of a dependent variate need not be weighted;
- (iv) the arithmetic means of the estimates of  $K$  and  $V$ , derived from the unweighted regressions (a) and (b), are the most reliable;
- (v) the apparent standard error of  $K$ , calculated from regression (a), is approximately equal to the true standard deviation, but the apparent standard error of  $V$  is greater than the true standard deviation.

Problem (iii) (estimating the magnitude of the residual variance about a first-order regression that can be attributed to a known experimental error) was solved as follows. It was assumed that the expected residual variance about regression (a),  $s^2$ , is given by the approximation:

Table 2.07

A Comparison of the Expected and Observed Residual  
Variances about a First-Order Regression of F on L

Time-course		10 <sup>6</sup> . residual variance	
simulated	expected	observed	
		mean	range
L(+) arabinose "low error"	0.202	0.224	0.031-0.480
L(+) arabinose "high error"	4.20	5.47	1.58 -8.49
D(+) glucose	105	229	99 - 460

The calculation of the "expected" residual variance  
is described on pp. 69 - 70.

$$s^2 = \frac{1}{n} \cdot \sum \left( \frac{f' \cdot c(f')}{t} \right)^2 \quad (2.35)$$

Hence an approximate value of  $s^2$  can be derived for a time-course of known variability.

Approximations were derived for each of the three sets of simulated time-courses, and are compared in Table 2.07 with the values actually observed. The expected and observed values are of the same order of magnitude. In consequence, expected residual variance about the regression is given by equation (2.35).

#### 4.05 Conclusion

The method based on a direct fit (Section 4.01) is the only one of the three elaborated in this Section that is theoretically valid. However, difficulties caused by the computations involved precluded its use. Therefore the method based on a bivariate fit (Section 4.02) was employed instead. It seems to give unbiased estimates of the carrier parameters, when the estimates are the means of those derived from regressions (a) and (b). It also indicates the standard deviations of the estimates, these being found from the standard errors of the coefficients of regression (a). But it does not incorporate an objective test of the goodness of fit of the permeation equation to a time-course. Nevertheless, the goodness of fit can be appraised by inspecting a plot of F against L, and by calculating the residual variance about regression (a).

Section 5

Summary

1. The Simple Membrane Carrier Hypothesis is described. The rate of net permeation of a mass of cells predicted from it is stated.

2. A permeation equation is derived for an experimental system that fulfils certain provisos.

3. Although the system studied does not fulfil these provisos, the permeation equation is an adequate approximation.

4. The permeation equation can be fitted to a time-course by a method based on two bivariate first-order regressions.  $K$  and  $V$  can be estimated from the fit.



## Chapter IV

## THE BULK DIFFUSION HYPOTHESIS

### Section 1

### Introduction

Ling (1962, 1966), and Fenichel & Horowitz (1963), have hypothesised that the rate of permeation of a cell by a non-electrolyte is governed by the rate at which the permeant diffuses through the protoplasm. In consequence, the kinetics of permeation are those of a process of uniformly slow, or bulk, diffusion.

These kinetics are given by a partial differential equation, and depend on the shape and size of the cell as well as on the experimental conditions. A general equation for the time-course of permeation is derived by integrating analytically the partial differential equation. Unfortunately, the integration can only be performed for a cell of constant volume and simple shape. No general equation exists for the time-course of permeation of a cell whose shape is that of a human erythrocyte in isosmolal solution, and whose volume varies as net permeation occurs.

The problem posed by the complex shape of the erythrocyte is solved if a cell of simple shape is an acceptable model of the erythrocyte. Although Roughton (1932), and Sha'afi, Rich, Sidel, Bossert & Solomon (1967), used the disc to represent the erythrocyte when they analysed its permeability to oxygen and water, respectively, they did not say why they chose this particular model. So the next Section of this Chapter includes an examination of the way in which the form of a time-course depends on

the shape of the cell. The examination suggests that cells of a simple shape have time-courses with similar forms. It is then argued that a disc of infinite radius is, with one important qualification, an adequate model of the erythrocyte.

Time-courses of permeation could have been measured in conditions in which the volume of the erythrocytes did not change. However, for the reasons mentioned in the General Introduction, these conditions were not adopted. Therefore the next Section of this Chapter also includes a brief discussion of how the errors introduced by changes in cell volume were minimised.

Section 3 comprises a description of the procedure used for fitting the chosen permeation equation to an experimental time-course, and for estimating the value of the characteristic parameters. The reliability of the procedure is proved in Section 4.

Section 2

The Permeation Equation

Crank (1956) has given permeation equations for three structures of constant volume and of ideal shape: they are a disc of infinite radius, an infinitely long cylinder, and a sphere. For example, equation (2.36) defines the time-course of permeation of a disc of infinite radius and uniform half-thickness  $e$ , suspended in an infinite volume of a solution of permeant of concentration  $x$ :

$$f = \frac{y}{x} = 1 - 2 \sum_{n=0}^{\infty} (\alpha_n \cdot \exp(\alpha_n \cdot z))^{-1} \quad (2.36)$$

where  $y$  = the mean concentration of intracellular permeant at time  $t$ ,

$$\alpha_n = (n + \frac{1}{2})^2 \cdot \pi^2,$$

$$z = Dt/e^2 \quad **$$

$D$  = the diffusion coefficient of intracellular permeant,

and the boundary condition is that  $y = 0$  when  $t = 0$ .

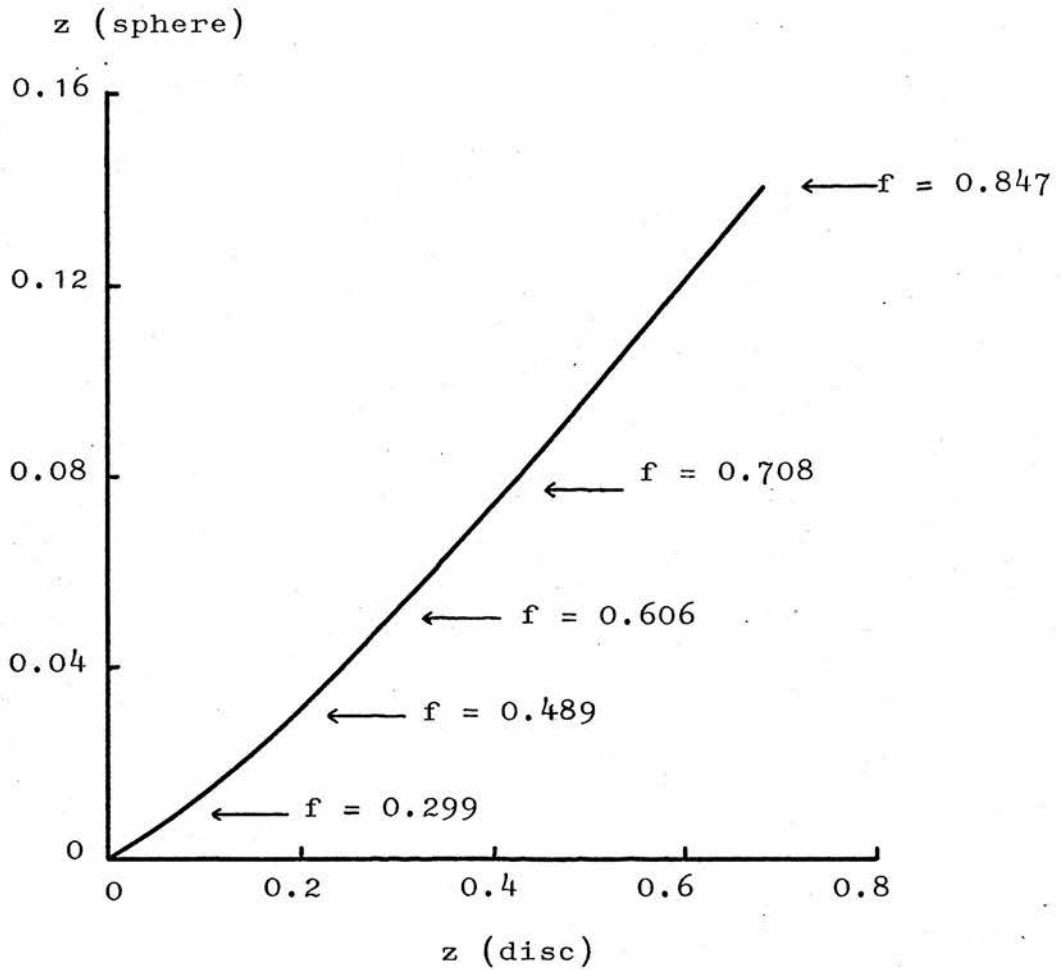
The equations for a cylinder and a sphere are similar, in that  $f$  is some function of  $z$ , where  $z$  equals  $Dt/e^2$ , and  $e$  is the finite dimension of the cell. Thus, for structures with these shapes,  $f$  is determined by  $D$  (the parameter characterising the rate-limiting mechanism),  $e$  (the dimension specifying the size of the cell), and  $t$ . For any one structure and permeant,  $D$  and  $e$  are constant, so that  $z$  is directly proportional to  $t$ .

It is pointed out in the preceding Section that the lack of an equation for the human erythrocyte is overcome if a cell of simple shape satisfactorily mimics the ery-

\*\* Crank represents  $Dt/e^2$  with the Greek symbol "tau".

Figure 2.03

The Plot of  $z$  (sphere) against  $z$  (disc)



The values of  $f$  that correspond to some of the values of  $z$  are shown.

throcyte. So, to establish whether the form of a time-course is much influenced by the shape of the cell, the forms of the time-courses for the three structures of ideal shape were compared.

The comparison was made by plotting against one another the values of  $z$ , for each structure, that correspond to a series of values of  $f$  ( $0 \leq f \leq 0.847$ ). These values of  $z$  were computed from the tables of  $f$  against  $z$  compiled by Berthier (1952), using the method for inverse non-linear interpolation described in Appendix A. The line relating  $z(\text{sphere})$  to  $z(\text{disc})$  is shown in Figure 2.03. Over most of its length, the line has a slope which does not vary greatly, but is not unity, and an intercept which is close to zero. The same is true of the lines relating  $z(\text{disc})$  to  $z(\text{cylinder})$ , and  $z(\text{cylinder})$  to  $z(\text{sphere})$ . Thus the form of a time-course does not seem to depend much on the shape of the structure. It follows that the permeation equation for one shape of cell fits closely to data observed with a different shape of cell. But the estimate of  $D/e^2$ , derived from the fit, is equal to the true value of  $D/e^2$  multiplied by a factor determined by the precise shapes of the two cells and the number and magnitudes of the values comprising the data. In the light of the first of these inductions, it is reasonable to follow the precedent of Roughton, and of Sha'afi et al., by taking the disc of infinite radius as a model of the erythrocyte.

The model must be used with at least one qualification.



The estimates of  $D/e^2$ , derived from different time-courses, deviate from their true values by factors determined by the number and magnitudes of the observed values of  $f$ . In consequence, the only estimates that are comparable to one another are those derived from time-courses with identical numbers and magnitudes of  $f$ -values.

The further problem, of allowing for the effect of changes in cell volume on the permeation equation, does not appear to have a quantitative solution. This stems from the lack of a lucid description of the way in which the net permeation of a non-electrolyte causes concomitant net permeation of water. The effect is presumably minimised by determining time-courses in conditions in which the changes are minimal. For this reason, the permeation equation was only fitted to experimental time-courses for which the extracellular non-electrolyte contributed less than 15% of the total extracellular osmolality. In these instances,  $f$  is nearly equal to  $f'$ . It was calculated from equation (2.06), which is derived in Chapter II, Section 1:

$$f = \frac{(m+x) \cdot f'}{m + x \cdot f'} \quad (2.06)$$

### Section 3      Fitting the Permeation Equation to a Time-Course

The permeation equation to be fitted to an experimental time-course is that for a disc of infinite radius, uniform half-thickness  $e$ , and constant volume:

$$\text{i.e. } f = 1 - 2 \cdot \sum_{n=0}^{\infty} ( \alpha_n \cdot \exp ( \alpha_n \cdot z ) )^{-1}$$

$$\text{where } z = Dt/e^2.$$

Strictly, the fit ought to be made by finding the value of  $D/e^2$  that minimises the sum of the squares of the (appropriately weighted) differences between the values of  $f$  which were observed and those which were calculated from the equation for the observed values of  $t$ . The goodness of the fit is indicated by the magnitudes and the nature of the distribution of these differences. If the magnitudes can be attributed to experimental error, and if the distribution is random, the equation is said to fit the time-course, and the best-fit value of  $D/e^2$  has been estimated. However, since this fitting procedure is complicated, and since equation (2.36) only approximates to the true permeation equation for the erythrocyte, a simpler, though less rigorous, procedure was devised.

The simple procedure is based on the assertion, first made in Section 2, that  $z$  is directly proportional to  $t$ . The values of  $z$  that correspond to the experimental values of  $f$  are computed, and related to the times,  $t$ , at which the values of  $f$  were attained. If, within the

limits of experimental error,  $z$  is directly proportional to  $t$ , the permeation equation fits that data when  $D/e^2$  equals  $\frac{dz}{dt}$ . The procedure is elaborated below.

Equation (2.36) expresses  $f$  as a function of  $z$ , and cannot be rearranged to give  $z$  as a simple function of  $f$ . In consequence, the value of  $z$  is inferred from that of  $f$  as follows. Berthier (1952) has tabulated  $f$  at intervals of  $z$ , although, according to Crank (1956), with an error which may exceed 1%. Therefore the tabulated values of  $f$  were checked, and the table was extended by direct calculation. The value of  $z$  is then calculated from that of  $f$  by inverse non-linear interpolation.

The hypothesis that  $z$  is directly proportional to  $t$  is tested in three ways:

- (a) A plot of  $z$  against  $t$  is inspected for marked deviations from direct proportionality. These can be rudely classified in the manner proposed by Matthews (1966).
- (b) The coefficients,  $p$  and  $q$ , of the unweighted first-order regression of  $z$  on  $t$  ( $z = p.t + q$ ), their standard errors,  $s(p)$  and  $s(q)$ , and the residual variance about the regression,  $s^2$ , are all calculated. It is reasonable to take  $z$  to be the dependent and  $t$  to be the independent variate, because  $z$  is computed from the error-containing variable,  $f$ . It is also assumed that  $z$  is normally distributed and of constant variance. Since it is suggested in Chapter III, Section 4.04, that the coefficient of variation of  $f$  does not vary much, the percentage changes in  $z$ , caused by changes in  $f$  of + 5% and -5%, were computed. They are shown

Table 2.08

The Changes in  $z$  caused by Changes of  $\pm 5\%$  in  $f$

$f$	+d	-d
0.160	10.4	11.8
0.252	10.3	10.0
0.357	10.5	9.9
0.451	10.3	9.8
0.551	10.7	9.9
0.650	11.6	9.9
0.739	13.4	11.9
0.837	18.4	14.2

+d and -d are the percentage changes in  $z$   
caused by changes of  $+ 5\%$  and  $-5\%$  in  $f$ .

in Table 2.08. Provided that  $f$  is not greater than 0.74, they are roughly equal and constant, and about twice the percentage change in  $f$ . This implies that each value of  $z$  ought to be given a weight inversely related to its value. Nevertheless, because a valid weighting function is difficult to devise, individual values of  $z$  are not weighted.

The significance of the difference between  $q$  and zero is ascertained by a Student's  $t$ -test, and the standard error of  $z$ , given by  $s$  (the square root of  $s^2$ ) is compared with that estimated from the expected coefficient of variation of  $f$ . If  $q$  does not differ significantly from zero ( $P > 0.05$ ), and if the standard errors are of the same order of magnitude, the hypothesis of direct proportionality is not contradicted.

- (c) The second-order regression of  $z$  on  $t$  ( $z = p_1 \cdot t + p_2 \cdot t^2 + q_2$ ) is calculated. A variance ratio test is used to determine whether the inclusion of the  $t^2$  term in the regression has removed a significant fraction of the residual variance ( $P < 0.05$ ). If it has not, the hypothesis is upheld.

If the hypothesis of direct proportionality passes all three tests, equation (2.36) is said to fit the time-course. The best-fit value of  $D/e^2$  is equated to the coefficient of the first-order regression, with no constant term, of  $z$  on  $t$  ( $z = p_0 \cdot t$ ). The standard error of  $p_0$  is an estimate of the standard error of  $D/e^2$ .



#### Section 4 The Reliability of the Fitting Procedure

The reliability of the procedure for estimating  $D/e^2$  from a time-course which would, but for its content of experimental error, be defined by equation (2.36) was proved in a manner similar to that described in Chapter III, Section 4.04.

A "perfect" time-course was prepared by choosing nine roughly equispaced values of  $f$  ( $0 \leq f \leq 0.837$ ), and computing the corresponding values of  $t$ , given that  $t = z \cdot e^2/D$ , and that  $D/e^2 = 1$ ;  $t$  ranged from 0 to 0.65. A simulated "experimental" time-course was constructed from the perfect one by multiplying each perfect  $f$  with a different member of an infinite series of pseudo-random numbers of mean unity and standard deviation 0.05 (the generation of the series is detailed on p. 64). The simulated time-course thus comprised the perfect values of  $t$ , and the randomised values of  $f$ . Ten different time-courses were constructed in this way.

The fit of equation (2.36) to each simulated time-course was tested by the simple procedure.

Test (a): in no instance did the plot of  $z$  against  $t$  deviate markedly from direct proportionality.

Test (b): in no instance did  $q$  differ significantly from zero. Since the coefficient of variation of  $f$  was 5%, the coefficient of variation of  $z$  ought, on the basis of the results in Table 2.08, to be about 12%. The mean of the nine values of  $z$  was 0.224, so the expected standard deviation of  $z$  was 0.027. In practice,  $s$  lay

between 0.014 and 0.044, and had a mean of 0.032. All these values are of the same order of magnitude as the expected value.

Test (c): in no instance did the inclusion of a  $t^2$  term improve the fit of a first-order regression of  $z$  on  $t$ .

In consequence, it was concluded that equation (2.36) fitted each time-course satisfactorily, so that  $D/e^2$  could be estimated.

The ten estimates ranged from 0.929 to 1.082, with standard errors of between 0.022 and 0.048. The estimates were not distributed asymmetrically (test of symmetry:  $P > 0.05$ ), and only one of the ten differed significantly from its "true" value of unity (Student's  $t$ -test;  $P > 0.05$ ). The mean of the ten was 1.012, the standard error of the mean being 0.018. Therefore this procedure gives an estimate of  $D/e^2$  which seems to be both accurate and precise.

## Chapter V A COMPARISON OF THE HYPOTHETICAL TIME-COURSES

### Section 1 The Simple Diffusion and The Simple Membrane Carrier Time-Courses

It is a commonplace that in some circumstances the rates of net permeation predicted from the Simple Diffusion and Simple Membrane Carrier Hypotheses are identical (see, for example, Bowyer, 1957). The rate predicted from the latter hypothesis is:

$$\frac{di}{dt} = w_o \cdot V \left\{ \frac{x}{K+x} - \frac{y}{K+y} \right\} \quad (2.30)$$

When  $K \gg x$ , this becomes:

$$\frac{di}{dt} = w_o \cdot \frac{V}{K} \cdot (x-y)$$

Equation (2.30) is empirically indistinguishable from equation (2.01), which defines the rate predicted from the Simple Diffusion Hypothesis:

$$\frac{di}{dt} = w_o \cdot P \cdot A \cdot (x-y) \quad (2.01)$$

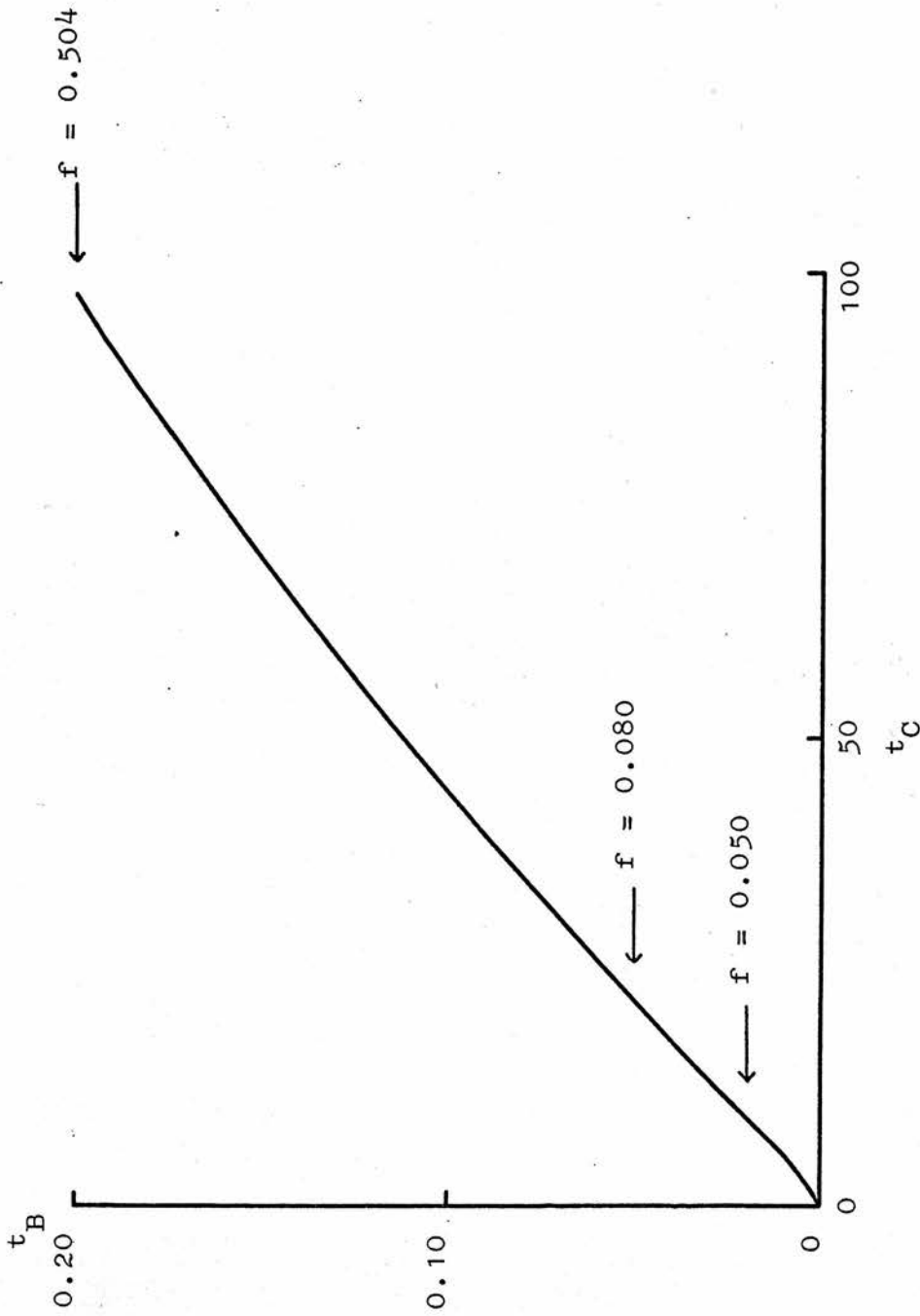
Therefore the conformity of a time-course to that of Simple Diffusion does not exclude the possibility that the kinetics of permeation are those of the Simple Membrane Carrier Hypothesis. Further experiments must be performed to justify the exclusion. These include the determination of time-courses at higher concentrations of extracellular permeant (which might be of the same order of magnitude as, and not much less than, the  $K$  of a possible carrier), in the presence of structural analogues of the permeant (which might compete with the permeant for a possible

carrier), and after the cells have been treated with agents, such as cupric ions, which are said to inhibit the action of some carriers.

Conversely, it is evident that if a time-course is fitted satisfactorily by equation (2.30) when  $K$  and  $V$  are finite, the rate of permeation is not limited by a mechanism of simple diffusion.

Figure 2.04b

The Plot of  $t_B$  against  $t_C$  when  $x = 20$



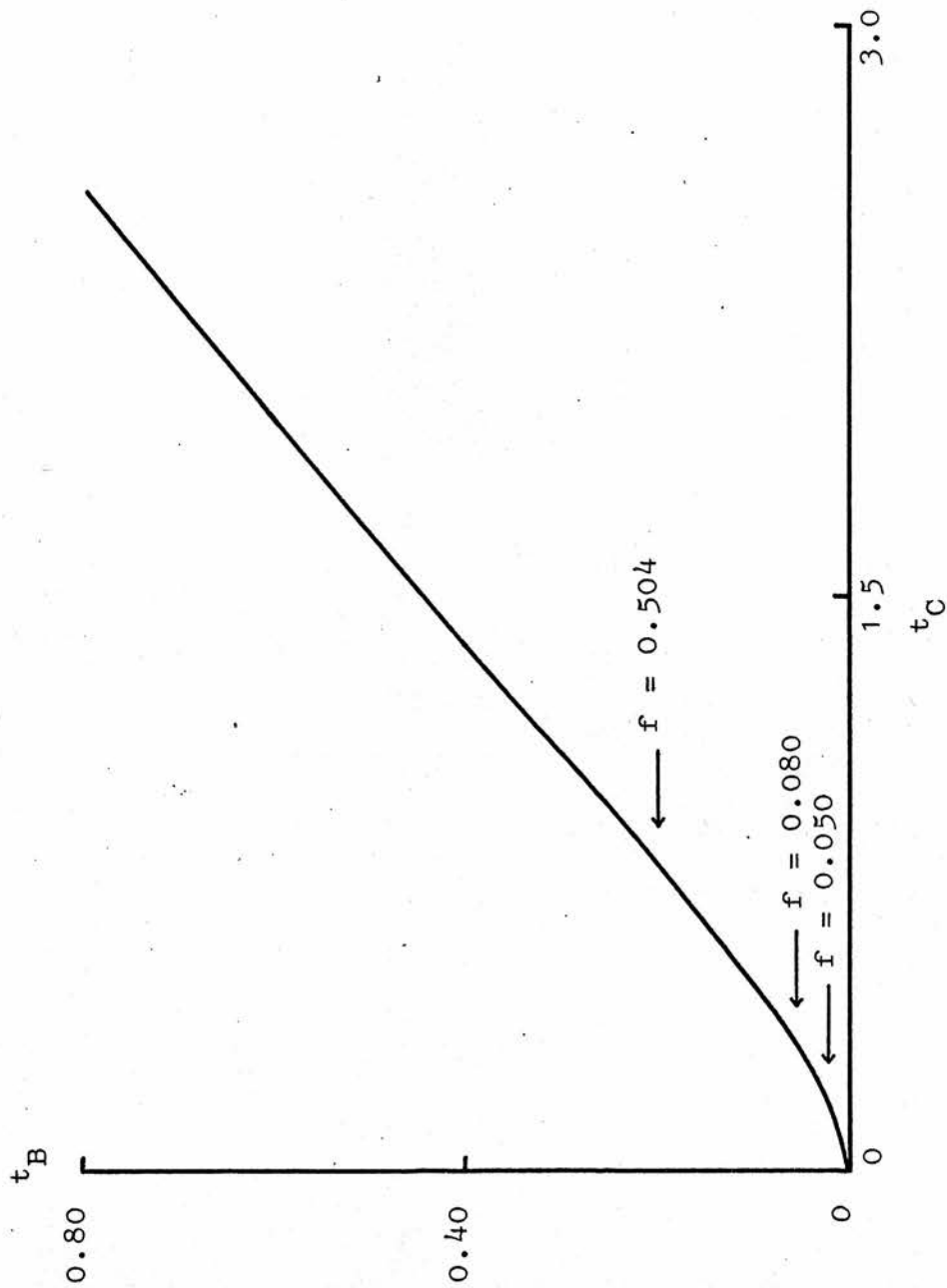
The values of  $f$  that correspond to 3 of the values of  $t_B$  are shown.

The calculation of  $t_B$  and  $t_C$  is described on p. 84.



Figure 2.04a

The Plot of  $t_B$  against  $t_C$  when  $x = 0.1$



The values of  $f$  that correspond to 3 of the values of  $t_B$  are shown.  
The calculation of  $t_B$  and  $t_C$  is described on p. 84.

## Section 2 The Simple Membrane Carrier and the Bulk Diffusion Time-Courses

Bronk & Fisher (1957) commented that the time-courses of permeation of the rat heart predicted from these hypotheses may be indistinguishable. Although the two permeation equations:

$$- \frac{(K+x)^2}{m.KV} \cdot \ln(1-f') - \frac{(K+m+x) \cdot (K+x) \cdot x}{m.KV} \cdot f' = t \quad (2.33)$$

$$\text{and} \quad f = 1 - 2 \sum_{n=0}^{\infty} (\alpha_n \cdot \exp(\alpha_n \cdot z))^{-1} \quad (2.36)$$

cannot be compared analytically, the forms of the time-courses are comparable. They are identical when the times for attaining any fractional penetration, predicted from the Simple Membrane Carrier and the Bulk Diffusion Hypotheses ( $t_C$  and  $t_B$ , respectively), are in direct proportion. A measure of the difference between the two forms is the extent to which the relationship between the two times,  $t_C$  and  $t_B$ , departs from one of direct proportionality.

The comparison for the erythrocyte is most easily made when  $m \gg x$ . In this circumstance, the volume of the cell is constant, so that  $f' = f$ , and equation (2.33) becomes:

$$- \frac{(K+x)^2}{K.V} \cdot \ln(1-f) - \frac{(K+x) \cdot x}{K.V} \cdot f = t \quad (2.37)$$

Equations (2.37) and (2.36) have been used to find  $t_C$  and  $t_B$ , given that  $D/e^2 = 1$ ,  $K = V = 1$ ,  $0.1 \leq x \leq 20$ , and  $0 \leq f \leq 1$ . In every case, of which those when  $x = 0.1$  and  $x = 20$  are illustrated in Figures 2.04a and 2.04b, the line relating  $t_B$  to  $t_C$  is sigmoid, its slope at first increasing, and

then decreasing slowly, as  $t_C$  and  $t_B$  increase. As  $x$  increases, the point on the line representing a fixed value of  $f$  moves in the direction of the region where, not only is  $t_C$  greater, but also the slope decreases slowly. In no case is  $t_B$  directly proportional to  $t_C$ .

Two practical considerations qualify the last statement. The first is that  $f$  generally falls between 0.2 and 0.8, and not 0 and 1.0; the second, that  $f$  is subject to experimental error. The constraint on  $f$  implies that there may be a range of values of  $x$  for which the lines appear to be nearly straight, and, on extrapolation, to pass hard by the origin. Error in  $f$  may then blur the distinction of these lines from ones which are straight, and pass through the origin. Both possibilities were confirmed as follows.

$t_C$  and  $t_B$  were calculated for a set of nine values of  $f$  ( $f=0, 0.160 \leq f \leq 0.837$ ), with  $0.1 \leq x \leq 20$ . Plots of  $t_B$  against  $t_C$  showed that:

- (i) when  $x = 0.1$ , the line is curvilinear, its slope increasing with  $t_C$ ;
- (ii) when  $x = 2$ , the line is sigmoid, its slope at first increasing, and then decreasing, as  $t_C$  increases;
- (iii) when  $x = 20$ , the line is again curvilinear, but with a slope which decreases as  $t_C$  increases.

The coefficients,  $p'$  and  $q'$ , with standard errors,  $s(p')$  and  $s(q')$ , of the first-order regression of  $t_B$  on  $t_C$  (i.e.  $t_B = p' \cdot t_C + q'$ ), for nine points, at each value

Table 2.09

The Regression of  $t_B$  on  $t_C$  atDifferent Values of  $x$ 

$x$	$10^3 \cdot p' \pm 10^3 \cdot s(p')$	$10^3 \cdot q' \pm 10^3 \cdot s(q')$	$10^2 \cdot \frac{s}{\bar{t}_B}$
0	373 $\pm 13$	- 45.6 $\pm 12.3$	10.0
0.1	322 $\pm 10$	- 41.8 $\pm 10.9$	8.9
1	121 $\pm 2$	- 18.0 $\pm 5.4$	4.7
2	59.3 $\pm 1.2$	- 3.88 $\pm 6.10$	5.5
3	35.2 $\pm 0.9$	4.63 $\pm 7.20$	7.0
5	16.5 $\pm 0.5$	14.3 $\pm 8.0$	9.0
8	7.61 $\pm 0.29$	21.6 $\pm 11.1$	10.7
20	1.46 $\pm 0.07$	30.7 $\pm 13.0$	12.7

$p' \pm s(p')$ , and  $q' \pm s(q')$  are the coefficients, with standard errors, of the unweighted first-order regression of  $t_B$  on  $t_C$  ( $t_B = p' \cdot t_C + q'$ ).  $s$  is the square root of residual variance about the regression, and  $\bar{t}_B$  is the mean of the nine values of  $t_B$ .

$x$  represents the concentration of extracellular permeant.

of  $x$ , are presented in Table 2.09. In every case, the coefficient of variation of  $p'$  is less than 5% and, when  $x > 1$ ,  $q'$  does not differ significantly from zero (Student's  $t$ -test:  $P > 0.05$ ). But the addition of a second-order term always improved the fit of a first-order regression to the points (analysis of variance:  $P < 0.05$ ). However, the square root of the residual variance about each first-order regression, expressed as a percentage of the mean of the nine values of  $t_B$ , is also given in Table 2.09. When  $0.1 < x < 5$ , its magnitude could be attributed to experimental error in  $f$ . Thus, when  $x$  is between, say, 1 and 3 (that is, one to three times  $K$ ), the forms of the two time-courses are, in practice indistinguishable. The exact range of values of  $x$  for which the apparent identity holds depends on the number, the magnitude and the accuracy of the values of  $f$  actually measured.

The comparison when  $x = 0$  deserves further comment, because equation (2.37) becomes

$$-\frac{K}{V} \cdot \ln(1-f) = t$$

This is the same as the permeation equation given by the Simple Diffusion Hypothesis. Therefore the forms of the time-courses predicted from the two diffusion hypotheses are quite different.

The negative correlation between  $p'$  and  $x$  is also important. It exemplifies that although equations (2.36) and (2.37) may both fit satisfactorily time-courses observed at two different concentrations of extracellular



permeant, the best-fit values of the parameters of at least one of the hypotheses must vary. For example, if permeation is limited by bulk diffusion, the same value of  $f$  is reached at a particular time whatever the concentration of extracellular permeant,  $x$ . The coefficients of the first order regression of  $F$  on  $L$ ,  $b'$  and  $a'$ , are also independent of  $x$ , and the apparent values of  $K$  and  $V$  calculated from them are directly proportional to  $x$ . Conversely, if permeation is carrier-mediated,  $K$  and  $V$  are constant, so that  $f$  depends on both  $x$  and  $t$ . The variation with  $x$  of the apparent value of  $D/e^2$  is the same as that of  $p'$ .

Chapter VI

SUMMARY

1. Three hypotheses of non-electrolyte permeation are considered. Each is characterised by a set of parameters, as follows:

- (i) The Simple Diffusion Hypothesis:  $P, A$ ;
- (ii) The Simple Membrane Carrier Hypothesis:  $K, V$ ;
- (iii) The Bulk Diffusion Hypothesis:  $D, e$ .

2. A unique permeation equation is derived from each of the hypotheses. These equations can only be applied to the human erythrocyte with reservations, which are discussed.

3. An observed time-course conforms to that predicted from a hypothesis if the permeation equation fits the time-course. Methods are described for fitting the permeation equations to a time-course, for appraising the goodness of the fits, and for estimating the best-fit values of the characteristic parameters.

4. In some experimental conditions, two of the three permeation equations may fit an observed time-course. But if time-courses are observed in different conditions, not more than one of the equations can be fitted to all the time-courses.

PART    THREE

EXPERIMENTAL METHODS AND MATERIALS

Chapter VII

INTRODUCTION

The approach employed in this investigation into some of the permeability properties of the erythrocyte is described in the preceding two Parts. Time-courses of permeation were determined using novel equipment, and analysed in terms of the three principal hypotheses of cell permeability. The novel equipment in question was a set of waisted centrifuge tubes, each of which could be "divided" into two compartments by the insertion of a plunger. These tubes permitted the separation of cells and extracellular medium in a manner such that the compositions of the intracellular and extracellular media at the moment of separation could be inferred from suitable estimates made at a later time. This Part describes how the time-courses were determined. It has six more Chapters, which are summarised below.

Chapter VIII is a general narrative of a typical experiment. It introduces and places in context the subjects of the succeeding Chapters. Thus in Chapter IX the preparation and properties of the principal media are listed, while Chapter X is devoted to the cells whose permeability was actually measured. Then in Chapter XI the design of the waisted centrifuge tubes and plungers and the accuracy and precision of the technique are delineated. The methods by which the chemical estimations were executed are the subject of Chapter XII. Finally, Chapter XIII is a summary.

Chapter VIII

A TYPICAL EXPERIMENT

A suspension of washed erythrocytes, of haematocrit about 40%, was prepared from whole blood by the procedure given in Chapter X, Section 1. On the morning of the experiment, the incubation medium was made up as described in Chapter IX, Section 3. It included inulin (which does not penetrate erythrocytes, and therefore acts as an "extracellular marker"), and permeant.

The experiment itself was conducted in a room whose temperature was controlled to  $\pm 1^{\circ}\text{C}$ . A 1ml aliquot of the suspension of washed erythrocytes was pipetted into each of a series of the waisted centrifuge tubes which are described in Chapter XI, Section 1. Then each tube was treated as follows. At time zero, 5 ml of incubation medium were rapidly injected into the tube from a syringe-pipette (B-D Cornwall Continuous Pipetting Outfit; Shandon Scientific Co. Ltd.). The tube was immediately inverted several times, and gently shaken, either manually or mechanically, for the appropriate period. Then it was centrifuged in an M.S.E. bench centrifuge for a total of 22 s. The centrifuge took about 5 s to reach its maximum speed (which was equivalent to a maximum relative centrifuge force of 3,000 x g), and was brought to rest by manual breaking in 7 s. Three seconds later, at time t, the tube was "divided" by the insertion of the plunger into its waist. Hence 25 s elapsed between the instants at which the centrifuge was started and the plunger was inserted. In this way, the contents of the lower com-



partment of the tube (that is, all the cells and some of the extracellular medium) were isolated from most of the extracellular medium (in the upper compartment of the tube) at the precisely - defined time,  $t$ .

The amount of intracellular permeant,  $i$ , and the concentration of extracellular permeant,  $x$ , at time  $t$  were calculated from estimates of the concentrations of permeant and inulin in the medium of the upper compartment, and of the amounts of permeant and inulin in the lower compartment. The calculation required that at time  $t$  the extracellular medium was homogeneous. It also required that the total amounts of the compounds in the two compartments did not then change, but not that permeation did not continue. Thus the estimates could have been made at any time after the plunger was inserted. However, although neither inulin nor any of the permeants bar glucose are metabolised by human erythrocytes, the "divided" tube was chilled in ice, and the estimates were made within 24 h.

The desired quantities were found as follows. An aliquot of the medium in the upper compartment was diluted and deproteinised. (In fact, there was only a little protein in this medium - see Chapter X, Section 2.05). The compartment itself was rinsed several times with distilled water, and the rinsings were discarded. Then the plunger was withdrawn, and the erythrocytes in the lower compartment were haemolysed by the addition of about 10 ml of distilled water to the tube. The contents

of the tube, which included all the permeant and inulin present in the lower compartment, were diluted to a suitable volume (generally 25 ml) and deproteinised. The procedures used to deproteinise the two solutions depended on the permeant being studied, and are given in Chapter XII. The methods by which the concentrations of permeant and inulin in the two protein-free solutions were estimated are also expounded in this Chapter.

Finally,  $w_o$ , the volume of intracellular water at isosmolality, was determined for another aliquot of the suspension of washed erythrocytes, as described in Chapter X, Section 2.04. The determination was always made at the temperature of the experiment, because  $w_o$  depends on temperature (Jacobs & Parpart, 1931).

The time-course of permeation could then be calculated. Each observation comprised a time,  $t$ , and the function of fractional penetration,  $f' (= i/x.w_o)$ , found at that time.

Chapter IX THE PREPARATION AND PROPERTIES OF THE MEDIA

Section 1

The Suspension Medium

The suspension medium was chosen to be of simple composition, and was based on that of Britton (1964). It was used for washing the erythrocytes, and for making up the "volume" and "incubation" media. It contained the following concentrations of "Analar-grade" salts in single-distilled water:

130.7 mM	NaCl
1.9 mM	$\text{NaH}_2\text{PO}_4$
8.1 mM	$\text{Na}_2\text{HPO}_4$

Its osmolality was measured with an Advanced "Uniform Universal" Osmometer (advanced Instruments Inc., Newton Highlands, Mass., U.S.A.), and was 0.268. Its pH at 21°C, measured with an E.I.L. Model 46A Vibret Laboratory pH Meter, was 7.34.

Two points deserve comment. Firstly, the composition of the medium does not resemble that of plasma. Therefore the erythrocytes were studied under well-defined, but not physiological, conditions.

Secondly, this medium was used even when time-courses were determined at different temperatures. Since the thermodynamic  $\text{pK}_a$  of  $\text{H}_2\text{PO}_4^-$  is 7.31 at 0°C and 7.20 at 25°C (Datta & Grzybowski, 1961), determinations made at different temperatures were also made at slightly different extracellular pHs.

Section 2

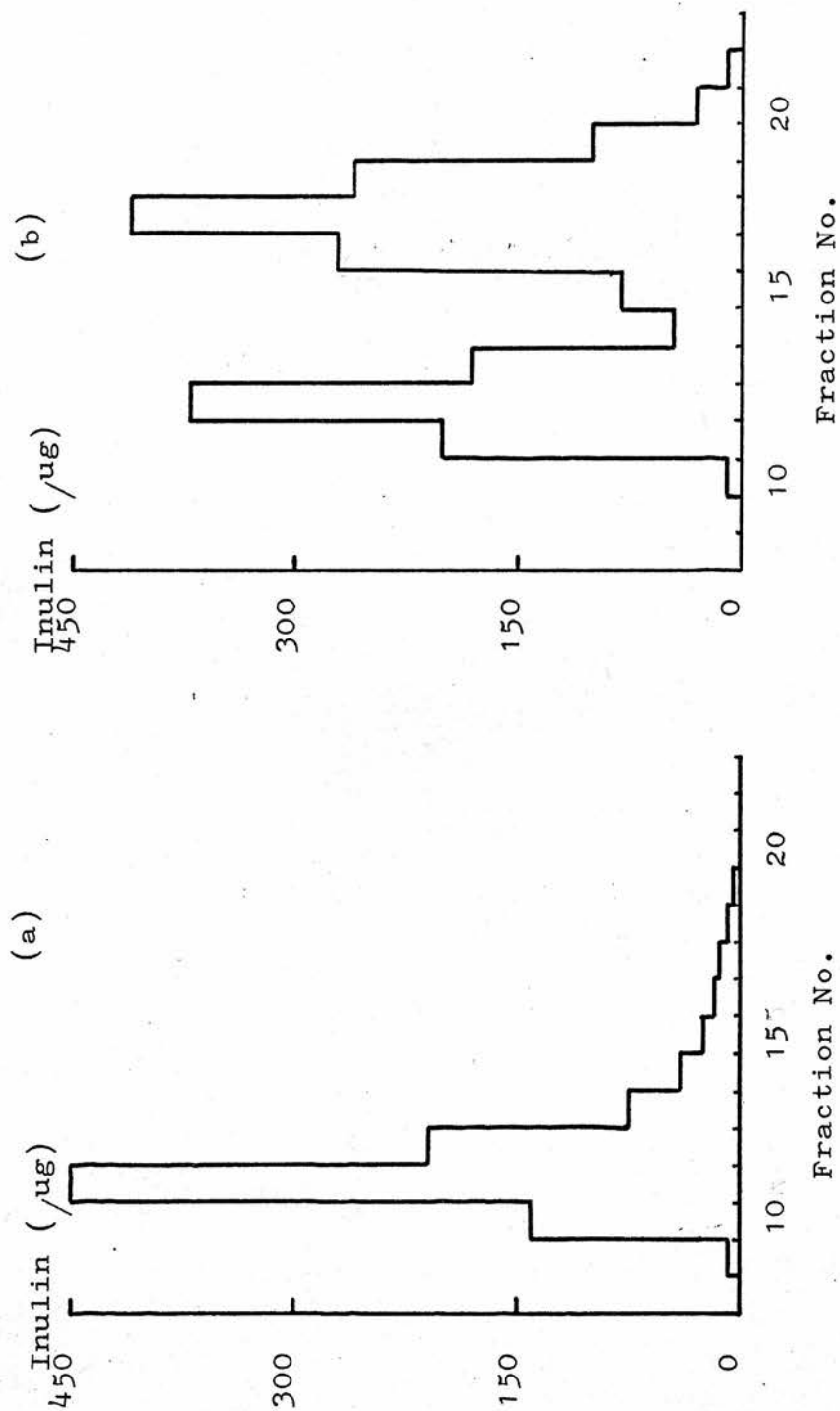
The Volume Medium

The volume medium was employed in the determination of the intracellular water content of the erythrocyte suspension, and in the preparation of the incubation medium. It contained 10 mg of inulin per ml of suspension medium. Throughout this study, B.D.H. "Laboratory Reagent" inulin, batch number 852250, was used. Since inulin is much less soluble in cold than in hot water, the volume medium was routinely made up by dissolving 1 g of inulin in about 60 ml of suspension medium at 60°C to 70°C, cooling the solution to room temperature, and then diluting it to 100 ml with suspension medium. The osmolality of the volume medium was defined as "isomolal". It was 0.268 (Advanced "Uniform Universal" Osmometer), and therefore indistinguishable from that of the suspension medium.

The inulin was supposed to act as an extracellular marker. Phelps (1965) has queried its reliability in this role, on the grounds that commercial products are polydisperse, containing polyfructosans of low molecular weight; are labile in media of mild acidity; and form metastable solutions. The last of these objections was discounted because the volume medium was clear even after it had been stored at 4°C for seven days. The possibility that the volume medium contained an appreciable concentration of fructose, or of fructosans of low molecular weight, was discounted for the following reason.

Figure 3.01a,b

The Chromatography of Inulin on Sephadex G-10



Chromatogram (a): 1 mg of inulin.

Chromatogram (b): 1 mg of inulin + 1 mg of fructose.



Three different preparations of the medium were chromatographed at room temperature on a column of dextran gel (Sephadex G- 10: molecular weight exclusion limit = 700). In each instance, 0.1 ml of volume medium was introduced onto the top of the column (1 cm internal diameter x 25 cm long), and then washed through the column with 0.05% (w/v) NaCl. Fractions of 1 ml were collected, and their concentrations of inulin and/or fructose were estimated by the method given in Chapter XII, Section 3. A typical elution pattern is shown in Figure 3.01a. For comparison: the pattern of Figure 3.01b was obtained when 1 mg of fructose was included in the 0.1 ml of volume medium chromatographed. It is clear that no fructose was detected in the volume medium. Moreover, in every instance, the percentage recovery in the eluate of inulin, or of inulin plus fructose, was between 98% and 102%. This indicates that the inulin did not come out of solution during chromatography.

Further evidence is presented in Chapter X, Section 2.03, on the efficacy of this particular batch of inulin as a marker of extra-erythrocyte water. Although an extracellular volume was never found by two independent methods, inulin was assumed to give a reliable measure of this quantity.

Section 3

The Incubation Medium

The incubation medium was prepared by dissolving permeant in volume medium. The permeants were:

L(+) arabinose: B.D.H. "Laboratory Chemical"

D(+) xylose: B.D.H. "Laboratory Reagent"

a - D(+) glucose: B.D.H. "Analar Grade"

b - D(+) glucose: Sigma Chemical Co.

meso-erythritol: B.D.H. "Biochemical"

thiourea: B.D.H. "Laboratory Reagent".

None was purified before use.

Most of the studies of permeability to glucose were carried out with solutions at mutatorotational equilibrium. a - D(+) glucose, dissolved in volume medium, was incubated at 60°C to 70°C for 10 min before the solution was cooled and diluted to the appropriate volume. When the permeation of a - or b - glucose alone was investigated, the incubation medium was made up 20 s before "time zero".

There was no appreciable difference between any permeant's milliosmolality, measured with the Advanced "Uniform Universal" Osmometer, and its millimolarity, calculated from the known composition of the incubation medium. Hence throughout this investigation milliosmolality was equated to calculated millimolarity.

Chapter X THE SUSPENSION OF WASHED ERYTHROCYTES

Section 1 The Preparation of the Suspension

1.01 The Source of the Erythrocytes

Whole human blood, as defined by Anon. (1968), was generously provided by the Edinburgh and South-East Scotland Blood Transfusion Service. It had been drawn from a healthy adult donor into acid-citrate-dextrose, and stored at 4°C to 6°C for not more than three days. The blood was of Group O, Rhesus Positive, or Group A, Rhesus Positive.

1.02 Washing the Erythrocytes

The erythrocytes were washed at room temperature. A 100 ml aliquot of whole blood was centrifuged at 800 x g for 15 min. The plasma, buffy coat and top layer of cells were sucked off, and 150 ml of suspension medium were added to the packed cells. The suspension was mixed thoroughly, and allowed to stand for 15 min. It was then centrifuged at 800 x g for 10 min. The supernatant and top layer of cells were sucked off, and 150 ml of suspension medium were added to the packed cells. The cycle of operations, from the mixing of the suspension to the addition of fresh medium, was repeated four more times. The suspension was stored at 4°C for not more than 24 h. It was then centrifuged at 800 x g for 10 min, and the supernatant was removed. The haematocrit of the packed cells was adjusted to about 40% with suspension medium, to give the "suspension of washed erythrocytes". No precautions were ever taken to preclude the contamination of this suspension by bacteria.

Table 3.01

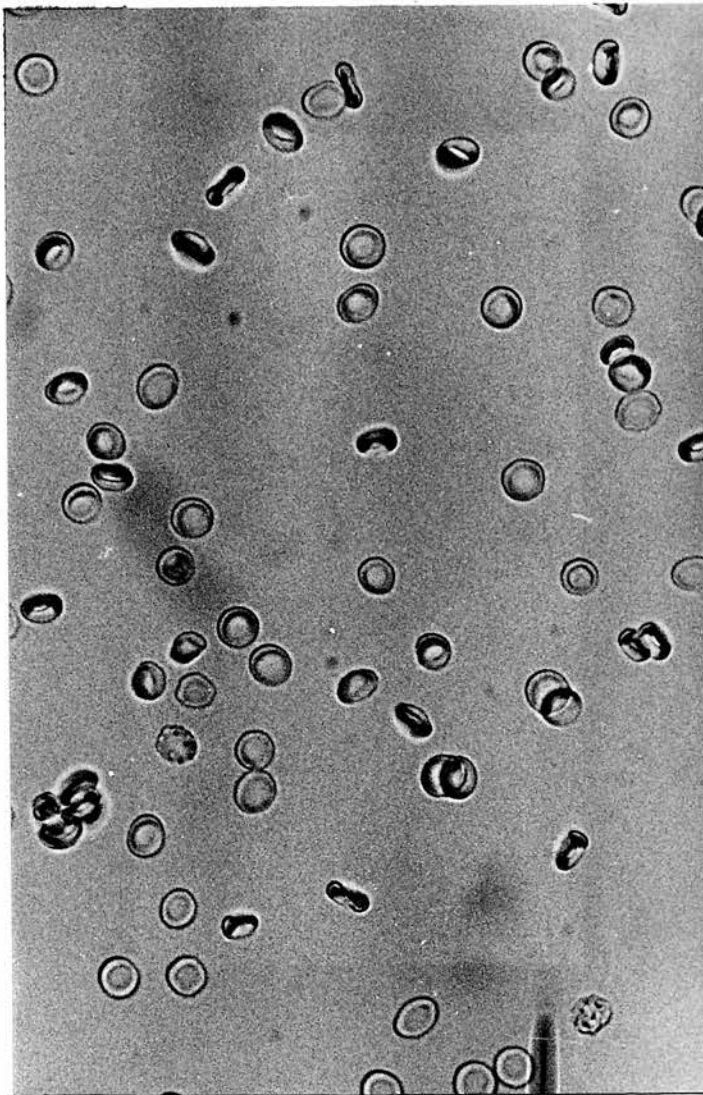
The Concentrations of some Intracellular Constituents  
in a Suspension of Washed Erythrocytes

Constituent	Concn. in intracellular water
Na <sup>+</sup>	30 mequiv./l
K <sup>+</sup>	130 mequiv./l
Cl <sup>-</sup>	100 mequiv./l
inorganic phosphate	6 mM
haemoglobin	7.6 mM

The preparation of the suspension of washed erythrocytes and methods for estimating the concentrations are given in Sections 1.02 and 2.02, respectively.

Figure 3.02b

The Appearance of the Erythrocytes After Washing



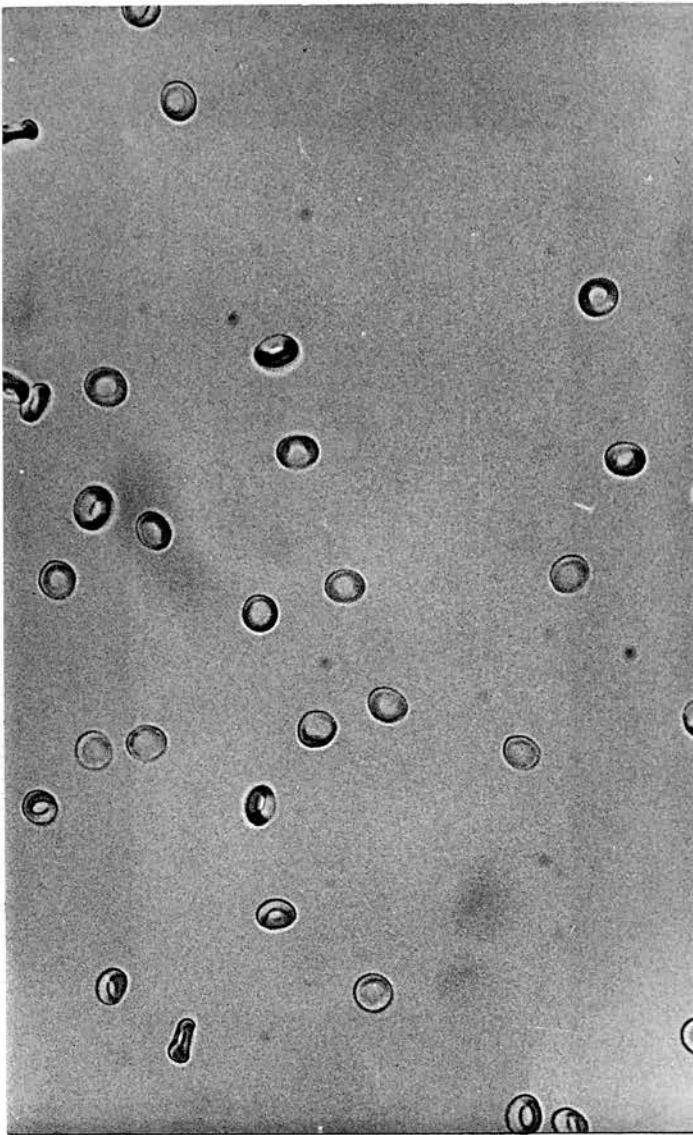
50  $\mu\text{m}$

A suspension of washed erythrocytes was mixed with Dacie's Fluid, and examined under a microscope at a magnification of 400 x.



Figure 3.02a

The Appearance of the Erythrocytes Before Washing



50  $\mu\text{m}$

Whole blood was mixed with Dacie's Fluid, and examined under a microscope at a magnification of 400 x.

Section 2

The Properties of the Suspension

2.01 The Appearance of the Erythrocytes

The erythrocytes were suspended in Dacie's Fluid (3% (w/v) tri-sodium citrate and  $\frac{1}{4}\%$  (w/v) formaldehyde), which "fixes" their shape (Darmady & Davenport, 1963), and examined under a microscope at a magnification of 400 x . Figures 3.02a and 3.02b show the appearance of cells taken, respectively, from a sample of whole blood and from the suspension prepared from that sample. They seem very similar.

2.02 The Chemical Composition of the Erythrocytes

The concentrations of some inorganic ions in the intracellular water of a typical suspension of washed erythrocytes are given in Table 3.01. They were derived thus.

The relative volumes of intracellular and extracellular water in the suspension were first determined by the method described in Section 2.04. The concentrations of  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Cl}^-$  and inorganic phosphate in the extracellular medium and in a haemolysate of the suspension were then estimated as follows:

- (i)  $\text{Na}^+$  and  $\text{K}^+$ : with an "EEL" flame photometer (Evans Electroselenium Ltd.).
- (ii)  $\text{Cl}^-$ : with an "EEL" Chloride Meter (Evans Electroselenium Ltd.). (A 10 ml aliquot of the haemolysate was first deproteinised by the addition of 2 ml of 10% (w/v) tri-chloroacetic acid).

- (iii) Inorganic phosphate: by the method of Hurst (1964). (A 10 ml aliquot of the haemolysate was first deproteinised by the addition of 2 ml of 10% (w/v) trichloroacetic acid).

Finally, the concentrations of the intracellular ions were calculated.

The concentration of haemoglobin in the intracellular water given in Table 3.01 was estimated with an "EEL" Haemoglobin Meter (Evans Electroselenium Ltd.).

Since human erythrocytes in vivo contain about 151 mequiv. of  $K^+$  and 21 mequiv. of  $Na^+$  per litre of intracellular water (Hill & Mills, 1961), the data in the Table suggest that the washed erythrocytes had lost some  $K^+$  and gained some  $Na^+$ . This is not unexpected, because the suspension medium contained no  $K^+$  or glucose, and the cells had been stored at  $4^{\circ}C$  for about 18 h. It is also probable that the cells had lost some lipid (Lovelock, 1955). Hence the "washed erythrocyte" is materially different from the erythrocyte in vivo.

It is important to establish that the washing procedure had removed all the glucose originally present in the whole blood, because the presence of endogenous glucose in a suspension of washed erythrocytes would lead to the misinterpretation of a time-course of monosaccharide permeation. Therefore the concentration of glucose in the extracellular medium was estimated by the method given in Chapter XII, Section 5. Since it was indistinguishable from zero, and since the "glucose blank" of the cells also appeared to be zero (Chapter XII, Section 5.07), the washed cell suspension was assumed to contain no glucose.

Table 3.02

The Volume of Extracellular Medium of a Suspension  
after Different Periods of Incubation

Time (h)	Mean volume (ml) $\pm$ SEM
0.2	18.6 $\pm$ 0.1
1.2	18.6 $\pm$ 0.1
2.2	18.5 $\pm$ 0.0
3.2	18.5 $\pm$ 0.2

Each mean volume is the average of four values.

Experimental details are given in Section 2.03.

## 2.03 The Determination of the Volume of Extracellular Medium

The volume of extracellular medium in a suspension of erythrocytes can be found by including an extracellular marker in the suspension medium. An ideal marker must neither penetrate the erythrocytes, nor be adsorbed onto their surface. Inulin was taken to satisfy these criteria, for the following reasons.

Firstly, had inulin penetrated the erythrocytes, the apparent volume of extracellular medium would have increased with time. Therefore this volume was measured at different times. Quadruplicate 20 ml volumes of a suspension of washed erythrocytes were each incubated at 25°C with 8 ml of volume medium. After 0, 1, 2 and 3 h, 5 ml of the incubated suspension were centrifuged at 3,000 x g for 10 min, the concentration of inulin in the resulting supernatant was estimated, and the volume of extracellular medium was calculated. The mean volumes, with standard errors, are given in Table 3.02. They did not increase with time. This evidence, together with that on the properties of inulin presented in Chapter IX, Section 2, was considered to show that no fructose-containing compounds penetrated the cells. However, the evidence is not conclusive, because the apparent increase in extracellular volume which such penetration would have caused could have occurred in the first 0.2 h, or could have been offset by a real increase in the volume of the erythrocytes due, for example, to a net influx of inorganic ions.



The second criterion is that inulin must not be adsorbed onto the cell surface. Its fulfilment would have been best demonstrated by measuring the same extracellular volume with a series of different markers. This was not done. However, Davson's (1964) summary of the use of inulin as an extracellular marker does not indicate that there is any evidence to suggest a binding of the polymer to cells like the erythrocyte.

#### 2.04 The Determination of the Volume of Intracellular Water

The volume of intracellular water in a suspension of washed erythrocytes at isosmolality was equated to the difference between the volumes of total water and extracellular water in the suspension.

The volume of total water per ml of suspension was found by drying 1 ml aliquots of the suspension to constant weight in an oven at 60°C (Macleod, 1932). Duplicates differed by not more than 0.3%. The water content, in ml, was identified with the weight, in g, lost by the suspension. It was usually about 0.880 ml.

The volume of extracellular water per ml of suspension was determined, in quadruplicate, by adding 2 ml of volume medium to 5 ml of the suspension, centrifuging the whole for 10 min at 3,000 x g, and estimating the concentration of inulin in the supernatant that resulted. Typically, the mean of the four determinations was 0.600 ml, and had a standard error of 0.002 ml.

Thus the volume of intracellular water per ml of

suspension,  $w_o$ , was generally about 0.280 ml. Repeated determinations on one suspension indicated that the standard deviation of  $w_o$  was approximately 0.003 ml.

A volume of intracellular water determined in this way may be subject to systematic errors. For example, Cook (1967) found that the weight lost by a suspension of erythrocytes depended on the temperature at which the suspension was dried, and reached a minimum, as opposed to a constant, value. He suggested that an observed decrease in weight was due not only to the evaporation of water but also to the oxidation of non-volatile material. The estimates of the volume of intracellular water which he derived for samples dried at 95°C and at room temperature differed from one another by no less than 2.5%. However, the data in Part Four that show an apparently complete equilibration of pentoses and meso-erythritol between the intracellular and extracellular water militate against the possibility of systematic errors in  $w_o$ .

## 2.05 The Stability of the Erythrocytes

The data on the chemical composition of the erythrocytes and on the apparent constancy of the extracellular volume which are given in Sections 2.02 and 2.03, respectively, suggest that the properties of the erythrocytes did not change much over a period of hours.

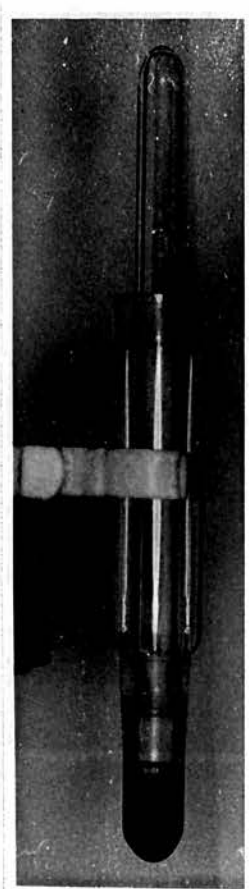
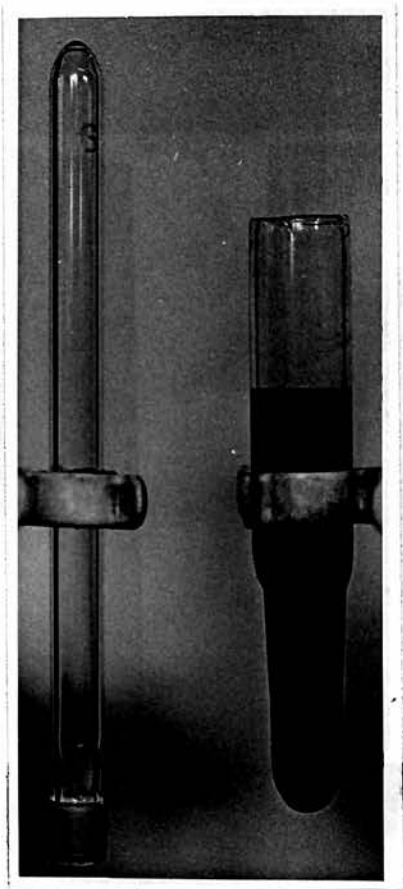
The percentage of the erythrocytes that haemolysed during an experiment like the one outlined in Chapter VIII was also determined. The haemoglobin contents of the extracellular medium in the upper compartment of a

divided centrifuge tube and of 1 ml of the suspension of washed erythrocytes were found by diluting aliquots of the medium and of the suspension with suitable volumes of 0.04% (v/v) ammonia solution, and measuring their extinctions at 540 nm. The percentage haemolysis was inferred from these contents.

Typically, there was 0.3% haemolysis after a 30 s incubation at 25°C with glucose, and 0.5% haemolysis after one of 1.5 h. Hence the decrease with time in  $w_0$  caused by haemolysis was ignored.

Figure 3.03

A Waisted Centrifuge Tube and Plunger



## Chapter XI THE WAISTED CENTRIFUGE TUBES AND PLUNGERS

### Section 1 The Design of the Tubes and Plungers

Figure 3.03 shows a "waisted" centrifuge tube and plunger, and a "divided" tube. Both tube and plunger were made of glass. The head of the plunger was ground so that it fitted tightly into the ground waist of the tube. The capacities of the "undivided" tube and of the upper and lower compartments of the "divided" tube are about 10.5, 6.0 and 0.8 ml, respectively.

Several considerations influenced the choice of these volumes. Ideally, the haematocrit of the cell suspension being incubated should be small. Both the decrease with time in the concentration of extracellular permeant, and the time for which the tube has to be centrifuged to corral all the cells in its lower compartment, are thereby reduced. This compartment should be full of cells, to increase the precision of the estimate of  $f'$ . Hence it ought to be of minimal volume. However, the compartment must contain permeant and inulin in amounts that can be estimated. Furthermore, as the haematocrit of the sedimented mass of cells increases, so does the period for which the tube has to be centrifuged at a given angular velocity. In consequence, the difference in composition between the bulk of the extracellular medium and that trapped within the mass also rises. These considerations indicate that the lower compartment should have a comparatively large volume.



Thus the volumes finally selected for the compartments are compromises. The lower compartment, of 0.8 ml, held amounts of permeant and inulin that were readily estimated by methods as sensitive as those described in Chapter XII. And it could be filled with a mass of cells of 50% haematocrit by centrifuging the tube at  $3,000 \times g$  for 22 s: a time which is not large when compared with that of most incubations - though of sufficient magnitude to preclude the study of the permeation of glucose at low concentration (see Part Four, Chapter XV).

## Section 2      The Accuracy of an Observation

Two of the factors that determined the accuracy of an observation,  $(t, f')$ , are not trivial, and are considered in this Section. The first is the extent to which the temperature of the suspension varied during incubation; and the second is the validity of an assumption introduced in Chapter VIII and alluded to in the preceding Section - the assumption that the extracellular medium was homogeneous at the instant the tube was "divided".

The temperature of the room in which the experiment was conducted never altered by more than  $0.5^{\circ}\text{C}$ , and that of the suspensions being incubated did not change by as much as this. Although the tubes were centrifuged in a standard M.S.E. bench centrifuge, the temperature of their contents did not increase greatly during centrifugation. For example, in an experiment carried out at  $2.0^{\circ}\text{C}$ , the temperature of these contents had risen to only  $2.4^{\circ}\text{C}$  after the 25 s centrifugation. Smaller increases were observed in experiments at higher temperatures. However, it must be emphasised that a rigid control of temperature is imperative, in view of the high  $Q_{10}$  of monosaccharide permeation (see Part Four, Chapter XV). Furthermore, an increase in temperature on centrifugation would affect short incubations more than long ones.

It is difficult to show conclusively that the extracellular medium was homogeneous at the instant the plunger was inserted. Had the mass of cells been of high haematocrit, and had it filled most of the lower compartment,

there could have been a significant difference between the concentrations of extracellular permeant in the two compartments at this instant. This difference would increase with the rate of net permeation of solute.

However, in the conditions used, any departure from homogeneity was probably small. The haematocrit of the mass of cells produced by centrifugation for 22 s at a maximum force of  $3,000 \times g$  was about 50%. Hence the volume of the medium surrounding the cells was roughly equal to that of the cells themselves, and an increase in  $f'$  would have been accompanied by a comparable decrease in the concentration of permeant in this part of the extracellular medium. The change in its concentration of inulin, which is determined by the change in the volume of the cells, would have been less. Furthermore, because the cells were not tightly packed, both permeant and inulin could have diffused between the bulk of the extracellular medium and that actually surrounding the cells. And the rate of diffusion of permeant in free solution is much greater than that into the human erythrocyte. This is exemplified by a calculation of Jacobs (1952): the time required for 90% saturation of the human erythrocyte by the rapidly - permeating urea is 500 ms, whereas had the same volume of water not been intracellular, it would have been 90% saturated after only 0.35 ms.

Nevertheless, it was in part to reduce the systematic error which inhomogeneity would cause that a tube was centrifuged for a relatively short time and the cells were not packed tightly.

### Section 3      The Precision of an Observation

The precision of an observation was largely determined by two factors: the precision of timing, and the precision of the methods of estimation. They are examined, in turn, below.

An incubation began when 5 ml of incubation medium were injected, from a syringe - pipette, into a tube holding 1 ml of erythrocyte suspension. Injection took about 1 s, and must have caused well - nigh perfect mixing of cells and medium. Thus the uncertainty in "time zero" was less than 1 s.

The procedure for centrifuging the tube and inserting the plunger is detailed in Chapter VIII. To recapitulate: centrifugation lasted a total of 22 s (5 s when the angular velocity was increasing, 10 s when it was constant, and 7 s when it was decreasing), and the plunger was inserted (at time  $t$ ) 3 s after the centrifuge had stopped. In practice, the procedure was generally executed in 23 to 27 s: if it took a time outwith this range, the tube was discarded and the incubation repeated.

Therefore the error in timing was small when compared with a total period of incubation of between 0.8 min and 25 min, so that "time",  $t$ , was to a first approximation an "independent", or error-free, variable. Nevertheless, attempts to determine the time-course of permeation of glucose of low concentration, when  $t$  lay between 0.5 and 2.0 min, gave observations of unacceptable precision, especially at the lower values of  $t$  (for example, at

$t = 0.5$  min, the coefficient of variation of  $f'$  was 15%). It was concluded that, in this circumstance, errors in timing and mixing were not negligible.

A value of  $f'$  is calculated from the amount of intracellular permeant per unit volume of intracellular water at isosmolality,  $i/w_0$ , and the concentration of extracellular permeant,  $x$ . The volume of the lower compartment and of the cells in it are both known. Therefore it is possible to derive a theoretical value for the coefficient of variation of  $f'$  from the coefficients of variation of the individual estimates of permeant and inulin concentration. Examples of such values for a typical L(+) arabinose time-course are given in Table 2.03 (facing p. 64 ). The volume chosen for the lower compartment was 0.8 ml, and that chosen for the cells in it was 0.4 ml. The coefficient of variation of an individual estimate of arabinose or inulin was taken to be 1.0% or 0.5%, respectively (see Table 3.12, facing p. 126 , and Table 3.07, facing p. 120 ), and  $w_0$  was assumed to be free of error. It is evident that the coefficient of variation of  $f'$  is much greater than those of the estimates from which it is derived. Hence, if the tubes are used in the way described in this Part, reliable values of  $f'$  can only be obtained if the methods of estimation are of great precision: especially as the theoretical coefficient of variation of  $f'$  is less than that realised in practice (see Part Four, Chapter XV).



## Chapter XII

## METHODS OF ESTIMATION

### Section 1

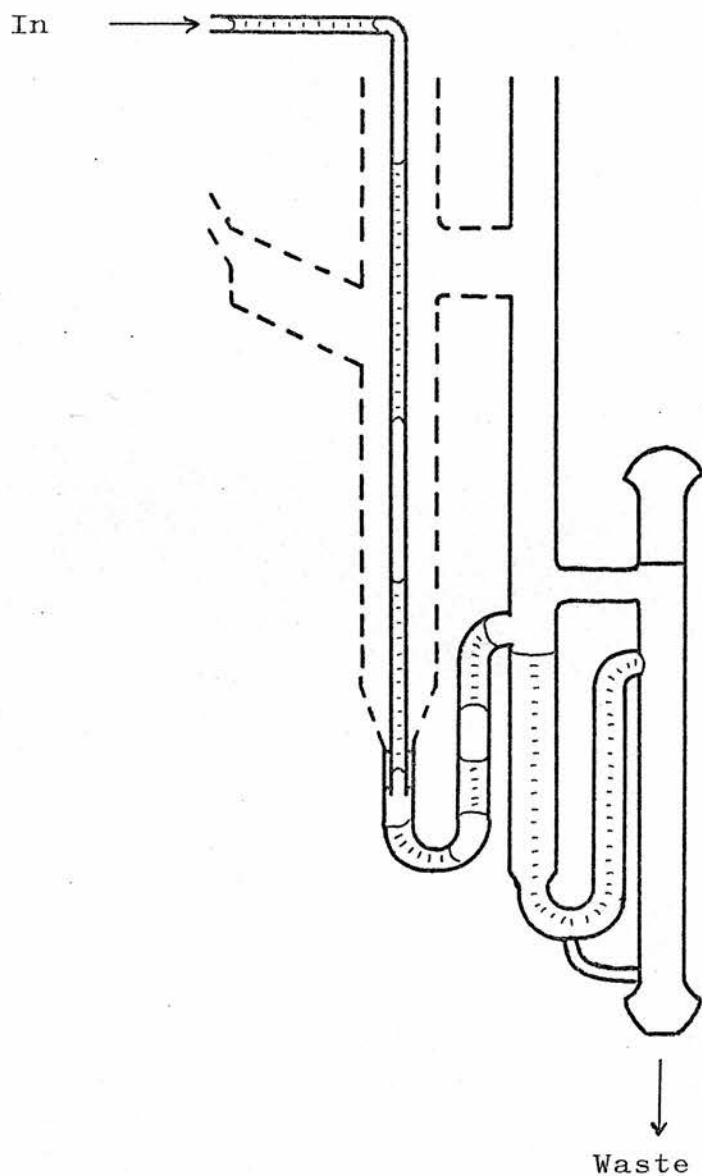
### Introduction

It is clear that the theoretical and practical approach employed in this study requires that all the chemical estimates be of great precision. These estimates were in practice made using an AutoAnalyser (Technicon Instruments, Ltd., Chertsey, Surrey). The principles of the AutoAnalyser were enunciated by Skeggs (1957). The way in which it is used in this laboratory is described in detail in the Dissertations of Gilbert (1963) and O'Brien (1969). Hence Section 2, which is devoted to the AutoAnalyser, only includes a discussion of the means whereby the reliability of the system was enhanced.

Sections 3 through 7 are concerned with the details of the methods by which inulin, glucose, pentoses, erythritol and thiourea, respectively, were estimated. These details include, for each compound: the assembly and calibration of the system; the deproteinisation of a sample; the accuracy and precision of an individual estimate; the apparent amount of endogenous compound in a haemolysate (the "blank" of the haemolysate for the compound); and the influence of other compounds on an estimate.

Figure 3.04

The Flow-Cell Modified for the Technicon Colorimeter



The parts of the original flow-cell that were omitted are shown by the dashed lines.

Section 2

The AutoAnalyser System

2.01 The Components of the System

All the estimations performed in this investigation are colorimetric. In consequence, each AutoAnalyser system comprised six independent units, connected in the following order: a sampler module (Mark I), a proportioning pump, mixing coils, a heating bath, a colorimeter and a recorder. A seventh unit, a dialyser, could have been added to the system to remove from a sample those macromolecules which would otherwise have interfered with an estimation procedure. Since this addition in general both reduces the sensitivity of an estimation procedure and increases the variance of an individual estimate, only samples which either contained no protein, or had been freed from protein, were presented to the system.

The flow-cell of the colorimeter, depicted in Figure 3.04, is a modification of the one actually supplied by Technicon. The modified flow-cell is a vertical optical cuvette, open to the atmosphere, with a horizontal inlet near the top and a vertical outlet at the bottom. When the stream of reaction mixture, which is segmented by bubbles of gas, enters the cuvette, the gas escapes to the atmosphere and the reaction mixture falls into the body of the cuvette. This stands in the light-path of the colorimeter, and the volume of liquid in it is kept constant by a siphon fitted to the outlet. In the original flow-cell, gas and reaction mixture separated

from one another in an open well which is similar in construction to, and precedes, the cuvette. Reaction mixture then siphoned into the cuvette. Since the half-time of replacement of the reaction mixture in the body of the cuvette by that of the incoming stream increases with the total volume of liquid in the flow-cell, by-passing the well increased the rate at which the per cent transmittance actually measured approached that of the incoming reaction mixture. Interaction between successive samples was thereby reduced. (A potential objection to the modified cell - that bubbles of gas might be swept into the light-path of the colorimeter - is without substance).

## 2.02 The Use of the System

The system was used in a manner designed to increase the reliability of an individual estimate.

The per cent transmittance (%T), rather than the extinction (E), of the reaction mixture in the cuvette was recorded. Therefore the scale of the ordinate on the chart paper of the recorder was linear, which permitted accurate interpolation.

The system was calibrated by recording the per cent transmittances of solutions of standard concentration. In general, three standards,  $S_1$ ,  $S_2$ ,  $S_3$ , gave a calibration curve of acceptable accuracy, provided that any departure from Beer's Law was small. Ideally, their per cent transmittances should have fallen between 60 and 20 units. Since any per cent transmittance can be read to within 0.1

Table 3.03

The Error in Extinction due to an Error  
Per Cent Transmittance

Per cent transmittance	Extinction	Error in extinction	Percentage error in extinction
10	1.0000	0.0043	0.43
20	0.6990	0.0021	0.30
30	0.5229	0.0014	0.27
40	0.3979	0.0011	0.28
50	0.3010	0.0009	0.30
60	0.2218	0.0008	0.36
70	0.1549	0.0006	0.39
80	0.0969	0.0006	0.62
90	0.0458	0.0004	0.87

The error in reading a per cent transmittance was taken as -0.1 units.



units, the error in the corresponding extinction which results from a reading error of this size varies with per cent transmittance in the way described in Table 3.03. It is evident that the percentage error in an extinction is close to its minimum value when  $60 \geq \% T \geq 20$ . In practice, however, per cent transmittances of less than about 45 units were often associated with unacceptable deviations from Beer's Law. Therefore the concentrations of the standards were chosen to give per cent transmittances of between 80 and 50 units.

Interaction between successive samples was eliminated by the insertion on the sampler turntable of cups filled with water between those filled with sample. Since the repeated estimation of a single sample sometimes showed that its per cent transmittance changed with time in a regular fashion, solutions were divided into sets of the three standards,  $S_1, S_2, S_3$ , and not more than five of the unknowns,  $U_1 \dots U_5$ . The solutions in a set were estimated in palindromic series:

for example  $S_1 \cdot S_2 \cdot S_3 \cdot U_1 \cdot U_2 \cdot U_3 \cdot U_4 \cdot U_5 \cdot U_5 \cdot U_4 \cdot U_3 \cdot U_2 \cdot U_1 \cdot S_3 \cdot S_2 \cdot S_1$  where a period represents a water-filled cup. In such a set, the concentration of each solution is represented by two values of per cent transmittance recorded at different times. But the mean time at which a pair of values was recorded is the same for all the solutions. Thus, when the mean of the pair of transmittances is used as a measure of concentration, the measurement on each solution in the set was, in effect, made at the same instant. This reduced errors due to a regular variation in per cent transmittance with time.

## 2.03 The Calculation of an Unknown Concentration

The concentration of a solution was always characterised by the logarithm of the product of the pair of per cent transmittances. This quantity is linearly related to the arithmetic mean of the pair of corresponding extinctions. The concentration was derived from the mean extinction, and the mean extinctions of the standards in the set.

In the earlier part of the investigation, in which pentose and inulin were estimated, the first-order regression relating the extinction to the concentration of the standards in a set was calculated. The concentration of a solution in the set was then found from the regression: a procedure which has been justified theoretically by Berkson (1950).

Latterly, when an "Olivetti Programma 101" desktop computer (P101) had become available, the first-order regression of concentration on extinction was used to predict an unknown concentration. The validity of the procedure is open to question because concentration, rather than extinction, approximates to the error-free variate. This objection was ignored because the coefficient of correlation between the two variates was never far from unity.

Further uncertainty was introduced when the P101 was programmed to execute the whole series of calculations automatically. The programme was only feasible because

Table 3.04

The Constants in an Expression Relating  $\log_{10} T$  to  $T$

---

Minimum value of $T$	$b_1$	$b_3$
0.30	0.864378204	0.357309584
0.35	0.866034052	0.341355475
0.40	0.867046806	0.329198563

$b_1$  and  $b_3$  are the constants in the expression:

$$-\log_{10} T = b_1 \cdot \left( \frac{1-T}{1+T} \right) + b_3 \cdot \left( \frac{1-T}{1+T} \right)^3$$

They were equated to the coefficients of the first-order regression, with no constant term, of  $-\log_{10} T$  on  $\left( \frac{1-T}{1+T} \right)$

and  $\left( \frac{1-T}{1+T} \right)^3$ , when  $T$  increased in increments of 0.005

from its minimum value to 1.00.

extinction (E) was expressed as a simple function of fractional transmittance (T). According to Zucker (1965):

$$\log_{10} T = 0.86304 \left( \frac{T-1}{T+1} \right) + 0.36415 \left( \frac{T-1}{T+1} \right)^3 + d$$

where  $d \leq 6 \times 10^{-4}$  when  $0.1 \leq T^2 \leq 10$ .

Since an observed fractional transmittance usually lay between 0.4 and 0.9, the coefficients  $b_1$  and  $b_3$  of the unweighted trivariate first-order regression, with no constant term:

$$-\log_{10} T = b_1 \cdot \left( \frac{1-T}{1+T} \right) + b_3 \cdot \left( \frac{1-T}{1+T} \right)^3$$

were computed, for T increasing in increments of 0.005 from 0.30, 0.35 and 0.40 to 1.00. They are presented in Table 3.04. When  $b_1 = 0.867046806$ ,  $b_3 = 0.329198563$ , and  $0.40 \leq T \leq 1.00$ , the maximum error in  $\log_{10} T$  is 0.15%. In consequence, the expression:

$$E = 0.867046806 \left( \frac{1-T}{1+T} \right) + 0.329198563 \left( \frac{1-T}{1+T} \right)^3$$

was taken to be an adequate description of the relation of extinction to fractional transmittance.

Both these procedures for finding an unknown concentration are based on the assumption that Beer's Law is obeyed. The conformity of data to the Law was always appraised by comparing the concentration predicted for each standard with its known value. When no predicted concentration differed by more than 1% from its true value, the assumption was said to be justified.

The assessment showed that the method for the estimation of glucose, which is summarised in Section 5, sometimes gave data which deviated markedly from the Law.

It was noticed that in these instances a satisfactory description of the relationship of extinction (E) to concentration (C) was:

$$\log E = a + b \cdot \log C$$

where a and b are the coefficients of the first-order regression of log E on log C. Therefore this regression was used to predict an unknown concentration.



Table 3.05

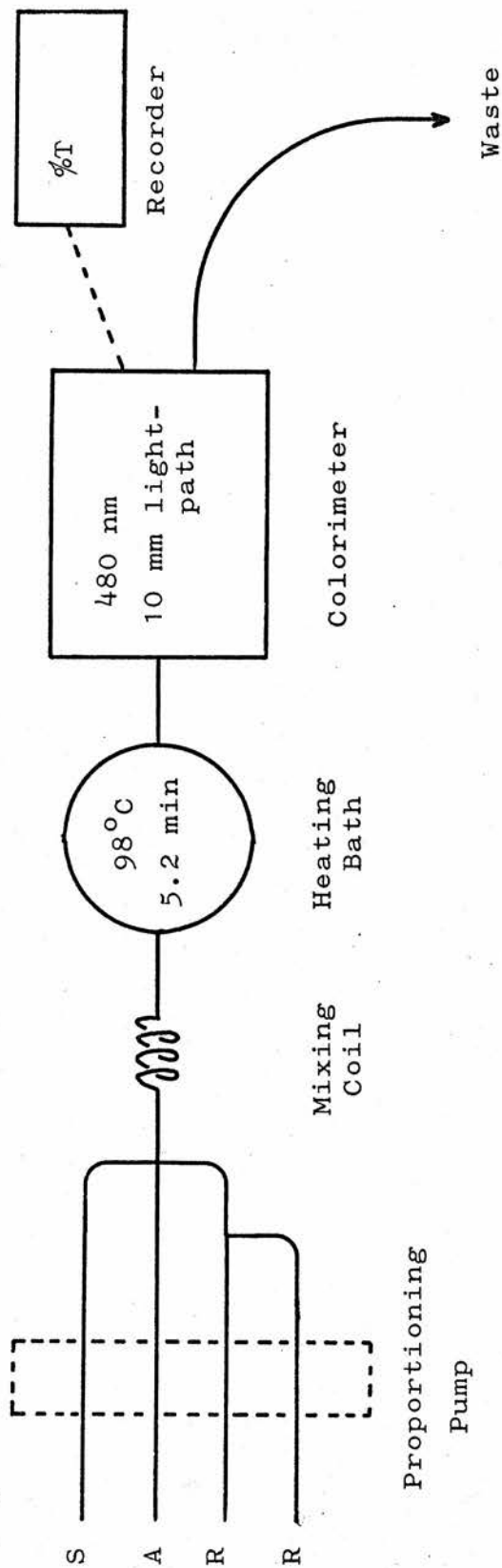
The Estimation of Inulin: the Calibration of the AutoAnalyser System

True concn. of inulin ( $\mu\text{g/ml}$ )	Per cent transmittance (% T)	Extinction (E)	Predicted concn. of inulin ( $\mu\text{g/ml}$ )
			$C_1$ $C_2$
10	80.5	0.094	9.94
20	66.8	0.175	20.13
30	55.9	0.253	29.94

$C_1$  and  $C_2$  are the concentrations of inulin predicted from the first-order regressions of E on C, and of C on E, respectively.

Figure 3.05

The Estimation of Inulin: the AutoAnalyser System



Flow rates: Sample(S): 1.2 ml/min  
Air (A): 0.6 ml/min  
Reagent (R): 3.9 + 1.6 = 5.5 ml/min  
Sampling rate: 40/h

Reagent: 0.1 g of resorcinol + 5 mg of ferric chloride in  
40 ml of water + 60 ml of conc. HCl.

## Section 3      The Estimation of Inulin

### 3.01    Introduction

The automated method used in this work was developed for the estimation of either raffinose or inulin by Gilbert (1963). Its basis is a manual method for the estimation of fructofuranosides, which Gilbert ascribed to S.W. Cole (unpublished work). These compounds react with ferric chloride and resorcinol in the presence of hot strong hydrochloric acid, to give a product with an absorption maximum near 480 nm.

Figure 3.05 shows the AutoAnalyser system; the reagent comprised 0.1 g of resorcinol and 0.005 g of ferric chloride dissolved in a mixture of 40 ml of distilled water and 60 ml of conc. HCl (specific gravity 1.18). The properties of the automated method that are relevant to this work are described below.

### 3.02    The   Calibration   of   the   System

The system was calibrated with three standard solutions, containing 10, 20 and 30  $\mu\text{g}$  inulin /ml. Table 3.05 illustrates a typical relationship between extinction at 480 nm (E) and concentration (C). It is not first order. However, its departure from first order is small: for example, the coefficient of correlation between the two variates is 0.99994. The table also gives  $C_1$  and  $C_2$ , the concentrations which are predicted from the first-order regression of E on C, and of C on E, respectively, to correspond to the observed extinctions. The greatest percentage difference between

a true and a predicted concentration is about 0.6. Moreover, the predicted concentration is equal to the true concentration when the latter is approximately 13 or 27  $\mu\text{g/ml}$ . Therefore the assumption of a first-order relationship between E and C results in a trivial error in an estimated concentration ( $C_2$ ), particularly when that concentration is close to either 13 or 27  $\mu\text{g/ml}$ .

### 3.03 The Deproteinisation of a Sample

Gilbert (1963) deproteinised with metaphosphoric acid a sample whose inulin concentration was to be measured. The pentose concentration of a sample deproteinised in this way cannot be estimated by his automated method, because the acid interacts with the reagent. He deproteinised a sample, before estimating its pentose concentration, by adding  $\text{CdSO}_4$  and  $\text{NaOH}$ , and mentioned that these reagents precipitated inulin as well as protein.

An attempt was made to find a protein precipitant that neither removes inulin from solution nor interferes with any method of estimation. Three protein precipitants, in the final concentration given, do not fulfil both criteria:

- (i) 10% trichloroacetic acid (Dawson, 1959).
- (ii) 1% Na tungstate / 0.067  $\underline{\text{N}}$   $\text{H}_2\text{SO}_4$  (Dawson, 1959).
- (iii) 5% perchloric acid.

$\text{ZnSO}_4$  /  $\text{NaOH}$  (Dawson, 1959) does, with certain qualifications.

Table 3.06

The Estimation of Inulin: the Recovery of Inulin  
from a "Deproteinised" Solution

Concn. of inulin in original soln. (mg/ml)	% recovery in "deproteinised" soln.
10	99.9
1	100.3
0.1	100.0
0.02	100.0

Duplicate 8 ml volumes were "deproteinised" with  
 $\text{ZnSO}_4/\text{NaOH}$  as described in Section 3.03.



The procedure which was finally adopted for a sample whose inulin, pentose, erythritol or thiourea concentration was to be measured, produced a clear, colourless supernatant from a haemolysate derived from 1 ml of a 40% suspension of washed erythrocytes. To the haemolysate, of volume either 8 or 10 ml, was added with continual shaking 1 ml of 10% (w/v)  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , followed by 1 ml of 2% (w/v) NaOH. Centrifugation of the mixture gave a supernatant whose concentration could be estimated satisfactorily. The results in Table 3.06 indicate that when duplicate 8 ml volumes of protein-free solutions containing either 10, 1, 0.1 or 0.02 mg of inulin per ml were treated with  $\text{ZnSO}_4$  and NaOH in this way, 100% of the inulin was recovered in the supernatants.

There are three qualifications. Firstly, when some batches of inulin were used, the recovery of the polymer in a supernatant was consistently less than 100%. These batches were rejected. Secondly, inadequate shaking of haemolysates, while the reagents were being added, resulted in variable recoveries. Thirdly, adding more than 1.2 ml of NaOH always reduced a recovery. Since a sample whose glucose concentration is to be estimated must contain at least this volume of NaOH (see Section 5.05), it is not possible to estimate accurately both the inulin and the glucose concentrations of a single  $\text{ZnSO}_4/\text{NaOH}$  supernatant.

#### 3.04 The Precision of an Individual Estimate

The standard deviation of an individual estimate was found by repeatedly estimating a solution whose inulin concentration was either 10, 20 or 30  $\mu\text{g/ml}$ . Table 3.07

Table 3.07

The Estimation of Inulin: the Precision of an Estimate

True concn. of inulin ( $\mu\text{g/ml}$ )	Mean estimated concn.of inulin ( $\mu\text{g/ml}$ )	Standard deviation of an individual estimate ( $\mu\text{g/ml}$ )
10	9.93	0.08
20	20.14	0.09
30	29.92	0.09

The concentration of inulin in each solution was estimated 21 times. The standard deviations of the estimates do not differ ( $P > 0.05$ ). Each mean estimated concentration differs from its true value ( $P < 0.002$ ).

is a summary of the data. Variance ratio tests suggested that at these concentrations the standard deviation of an individual estimate was constant ( $P > 0.05$ ). Student's t-tests showed that each mean estimated concentration differed significantly from its true value ( $P < 0.002$ ), which supports the conclusion that E is not linearly related to C. In consequence, samples of unknown inulin concentration were diluted until their concentration was about 27  $\mu\text{g/ml}$ .

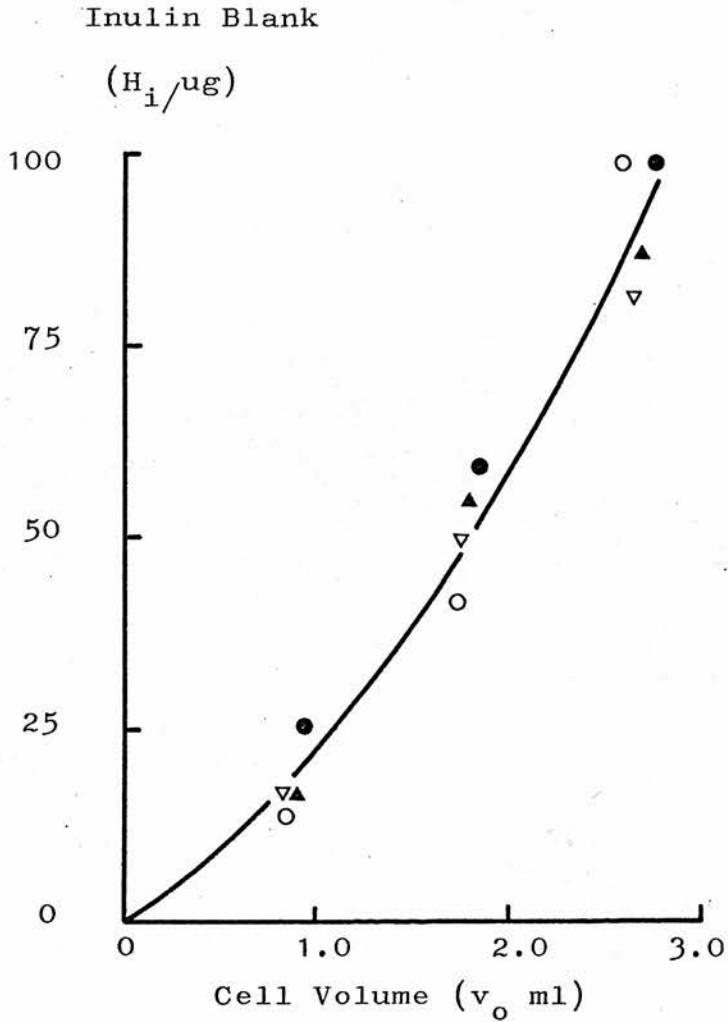
### 3.05 The Inulin Blank of a Haemolysate

Gilbert (1963) observed that the supernatant of a deproteinised rat heart homogenate appeared to contain a variable amount of inulin, which he called the inulin blank of that heart. The size of the blank depended on the protein precipitant used, perchloric acid and trichloroacetic acid giving larger blanks than did metaphosphoric acid. In consequence, the inulin blank of a haemolysate derived from a suspension of washed erythrocytes was evaluated, as follows.

The total isosmolal volume of cells per ml of a suspension of washed erythrocytes,  $v_0$  ml, and the volume of water in these cells,  $w_0$  ml, were determined by the method described in Chapter X, Section 2.04. A known volume of suspension (which was between 0 and 6 ml) was incubated for a few minutes at room temperature with 1 ml of suspension medium containing 650  $\mu\text{g}$  of inulin. The cells were haemolysed by the addition of a distilled water, and the haemolysate was mixed with  $\text{ZnSO}_4$  followed

Figure 3.06

The Inulin Blank of a Haemolysate



The determination of an inulin blank is described in Section 3.05.

Each symbol represents a different donor's erythrocytes.

The line through the points is defined by equation (3.01).

by NaOH. The mixture was diluted to 25 ml with distilled water and centrifuged. Finally, the inulin concentration of the supernatant ( $C_i$  /ug/ml) was estimated. The inulin blank of the haemolysate ( $H_i$  /ug) is equal to the difference between the apparent and the true contents of inulin in the haemolysate:

$$\text{i.e. } H_i = (25 - (v_o - w_o)) \cdot C_i - 650 \text{ /ug.}$$

Figure 3.06 shows the relationship between  $H_i$  and  $v_o$ , for four different donors' erythrocytes. The relationship is not first-order, a variance ratio test indicating that the addition of a term in  $v_o^2$  to the regression of  $H_i$  on  $v_o$  removed a significant fraction of the residual variance about the regression ( $P < 0.01$ ). The second-order regression is:

$$H_i = -0.70 + 16.40 \cdot v_o + 6.75 \cdot v_o^2 \quad (3.01)$$

and the residual sum of squares about it is 448.7.

An equation of the type  $H_i = \bar{A} \cdot \exp(\bar{B} \cdot v_o)$ , which must be satisfied by the point (0,0), was also fitted to the data: the unweighted first-order regression of  $\log H_i$  on  $v_o$  was calculated, and  $\bar{A}$  and  $\bar{B}$  were derived from its coefficients. The equation is

$$H_i = 8,00 \cdot \exp(0.94 \cdot v_o) \text{ /ug} \quad (3.02)$$

and the residual sum of squares is 1116.5. Therefore equation (3.01) seems to give the better fit, and was used to calculate the inulin blank of a haemolysate derived from a known volume of erythrocytes.

The dependence of the size of an inulin blank on the protein precipitant used was briefly investigated.



Table 3.08

The Estimation of Inulin: the Interference of Pentose

Inulin concn. of sample ( $\mu\text{g/ml}$ )	Increase in apparent inulin concn. ( $\mu\text{g/ml}$ ) when the sample contained arabinose of concn.:		
	50 $\mu\text{g/ml}$	100 $\mu\text{g/ml}$	150 $\mu\text{g/ml}$
10	0.07	0.12	0.20
20	0.06	0.14	0.20
30	0.09	0.13	0.18

Each inulin concentration was estimated once.

The first-order regression of the increase in apparent inulin concentration,  $Z_i$   $\mu\text{g/ml}$ , on arabinose concentration,  $C_p$   $\mu\text{g/ml}$ , is:

$$Z_i = 0.00127 (\pm 0.00005) \cdot C_p + 0.004 (\pm 0.005)$$

Aliquots of one haemolysate were deproteinised with either metaphosphoric acid of final concentration 2% (w/v), or  $\text{ZnSO}_4/\text{NaOH}$ . The two blanks were of similar size (21.7 and 22.8  $\mu\text{g}$  inulin /ml cells, respectively).

It was concluded that since in practice a  $\text{ZnSO}_4/\text{NaOH}$  blank would contribute less than 1% of the inulin which appeared to be present in the lower compartment of a waisted centrifuge tube, its size could be calculated from equation (3.01) with sufficient accuracy.

### 3.06 The Interference of Pentose

Gilbert (1963) found that the presence of pentose in a sample increased its apparent raffinose concentration, and that the increase was a minimum when the concentration of resorcinol in the reagent was 0.1% (w/v).

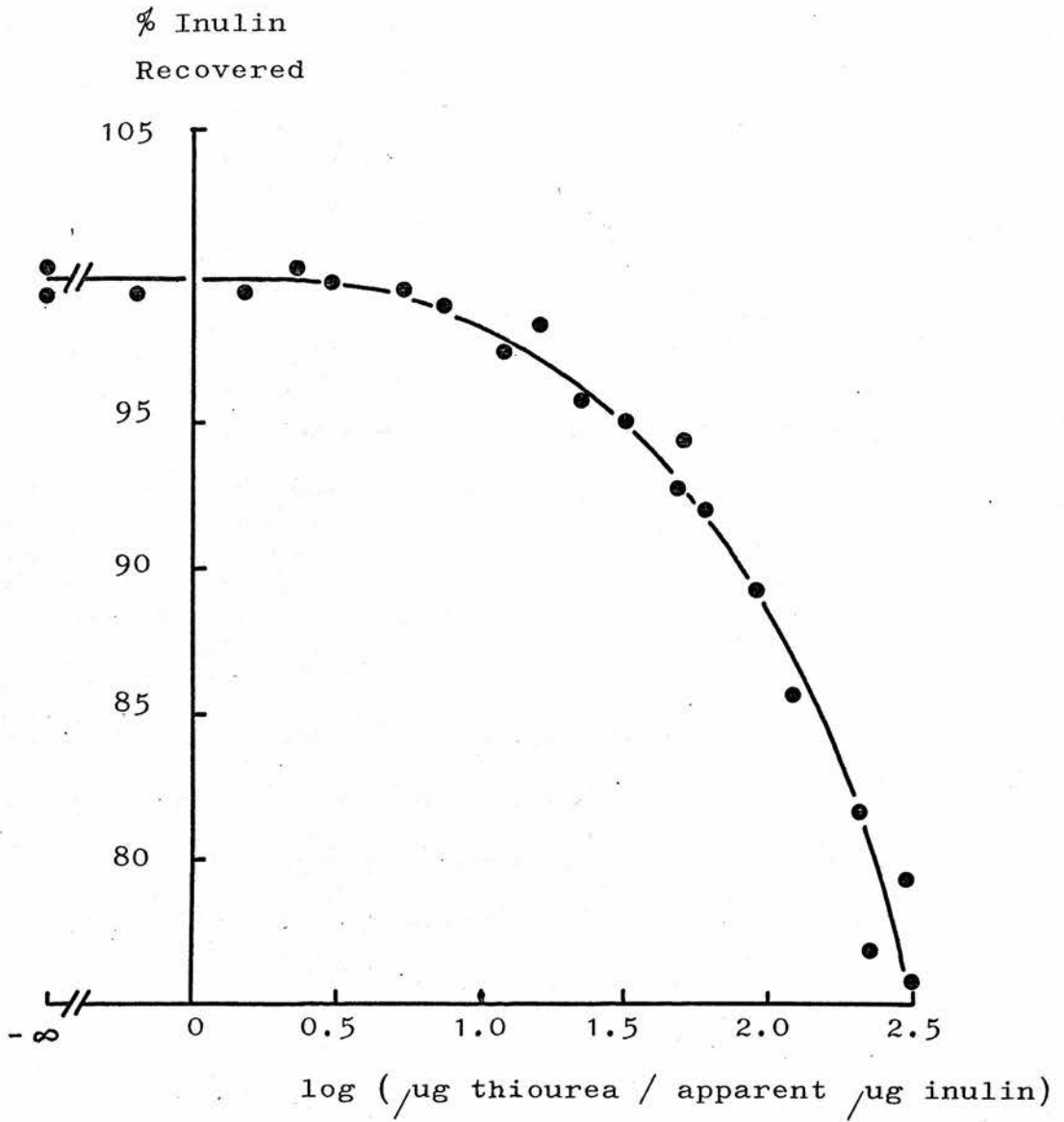
The magnitude of the increase in an apparent concentration of inulin that was caused by pentose was established by estimating the inulin concentration of samples of known inulin and L(+) arabinose concentrations. The results are presented in Table 3.08. They demonstrate that, in the concentration ranges explored, an increase ( $Z_i$   $\mu\text{g}/\text{ml}$ ) was independent of inulin concentration, and directly proportional to arabinose concentration ( $C_p$   $\mu\text{g}/\text{ml}$ ). The first-order regression of  $Z_i$  on  $C_p$  is:

$$Z_i = 0.00127 (\pm 0.00005) \cdot C_p + 0.004 (\pm 0.005)$$

Hence an estimated concentration of inulin was corrected by subtracting ( $0.00127 \cdot C_p$ )  $\mu\text{g}/\text{ml}$ . In practice a correction was small, being about 0.4  $\mu\text{g}/\text{ml}$  when the

Figure 3.07

The Effect of Thiourea on an  
Estimate of Inulin Concentration



The line through the points was drawn by eye.

Table 3.10

The Estimation of Inulin: the Interference of Erythritol

Inulin concn. of sample ( $\mu\text{g/ml}$ )	Increase in apparent inulin concn. ( $\mu\text{g/ml}$ ) when the sample contained erythritol of concn.:		
	50 nmol/ml	100 nmol/ml	150 nmol/ml
10	0.0	-0.1	0.0
20	0.0	0.0	0.0
30	0.0	-0.1	0.1

Each inulin concentration is the mean of two estimates.

Table 3.09

The Estimation of Inulin: the Interference of Glucose

Inulin concn. of sample ( $\mu\text{g/ml}$ )	Increase in apparent inulin concn. ( $\mu\text{g/ml}$ ) when the sample contained glucose of concn.:			
	50 $\mu\text{g/ml}$	100 $\mu\text{g/ml}$	150 $\mu\text{g/ml}$	200 $\mu\text{g/ml}$
10	0.96	1.43	2.57	2.74
20	1.00	1.75	2.54	3.16
30	0.49	1.35	2.35	2.68

Each inulin concentration was estimated once.

The first-order regression of the increase in apparent inulin concentration,  $Z_i$   $\mu\text{g/ml}$ , on glucose concentration,  $C_g$   $\mu\text{g/ml}$ , is:

$$Z_i = 0.0148 (\pm 0.0008) \cdot C_g + 0.057 (\pm 0.099)$$



ratio of the amounts of pentose to inulin had its maximum value of 10  $\mu\text{g}$  pentose /  $\mu\text{g}$  inulin.

### 3.07 The Interference of Glucose

The apparent concentration of inulin in a sample depended on glucose concentration in the manner shown in Table 3.09. The first-order regression of  $Z_i$ , the apparent increase in inulin concentration in  $\mu\text{g}/\text{ml}$ , on  $C_g$ , the concentration of D(+) glucose in  $\mu\text{g}/\text{ml}$ , is:

$$Z_i = 0.0148 (\pm 0.0008) \cdot C_g + 0.057 (\pm 0.099)$$

Therefore the estimated concentration of inulin in a sample which contained  $C_g$   $\mu\text{g}$  glucose /  $\text{ml}$  was corrected by the subtraction of  $0.0148 \cdot C_g$   $\mu\text{g}/\text{ml}$ . In practice a correction never exceeded 0.5  $\mu\text{g}/\text{ml}$ .

### 3.08 The Interference of Erythritol

Table 3.10 demonstrates that the apparent concentration of inulin in a sample was unaffected by the presence of erythritol when the ratio of the amounts of the two compounds was not less than 10  $\mu\text{g}$  of inulin to 150 nmol of erythritol. In practice the ratio always exceeded this value.

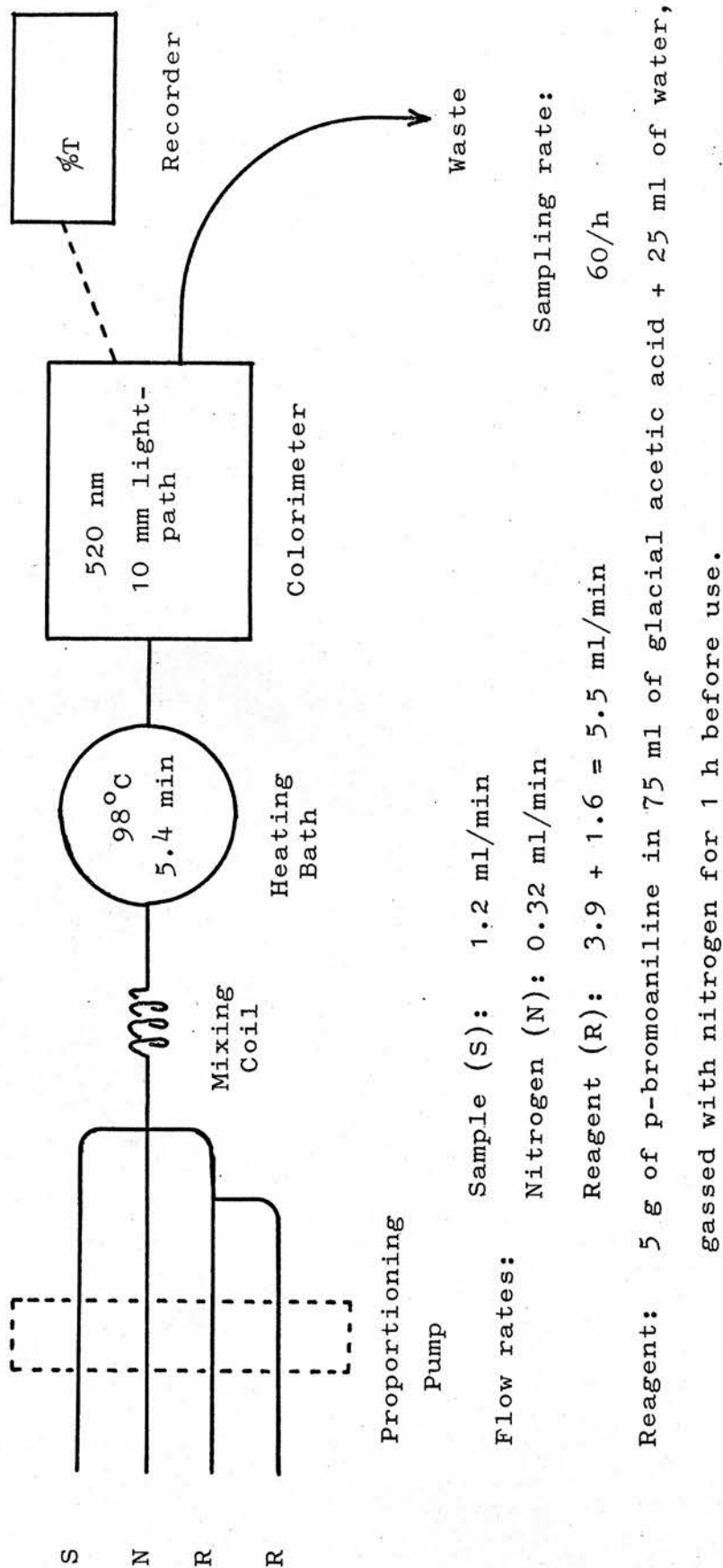
### 3.09 The Interference of Thiourea

The inclusion of thiourea in a sample decreased the apparent amount of inulin in that sample. The relationship between the percentage decrease in the amount of inulin and the amount of thiourea present is best illustrated by Figure 3.07. The line through the points was drawn by eye, because no simple mathematical expression could be fitted to the two variates. The graph was used to correct an estimate of inulin concen-

tration. In practice,  $\log (\mu\text{g thiourea} / \text{apparent } \mu\text{g inulin})$  was never more than 1.5.

Figure 3.08

The Estimation of Pentose: the AutoAnalyser System



## Section 4

## The Estimation of Pentose

### 4.01 Introduction

The automated method used in this work is that of Gilbert (1963); it is based on a manual method described by Roe & Rice (1948). In both methods, pentose is converted by hot acetic acid to furfural, which then combines with p-bromoaniline to give a coloured product with an absorption maximum near 520 nm. The methods are not specific for a particular pentose or even for pentoses in general, because prolonged heating can cause the formation of furfural from other precursors.

The arrangement of the AutoAnalyser system is depicted in Figure 3.08. Each litre of the reagent was prepared by dissolving 50 g of p-bromoaniline in 750 ml of glacial acetic acid, adding 250 ml of distilled water, and bubbling nitrogen gas through the whole for at least one hour.

Only those properties of the automated method that were established in this investigation are discussed below.

### 4.02 The Calibration of the System

The system was generally calibrated with three standard solutions, of pentose concentration 30, 45 and 60  $\mu\text{g/ml}$ . Extinction at 520nm (E) was assumed to be linearly related to concentration (C  $\mu\text{g/ml}$ ), because, in different experiments,  $d^2 E/dC^2$  (the second differential of E with respect to C) was either positive or negative, and always close to zero. The extinction

Table 3.12

The Estimation of Pentose: the Precision of an Estimate

True concn. of pentose ( $\mu\text{g/ml}$ )	Mean estimated concn. of pentose ( $\mu\text{g/ml}$ )	Standard deviation of an individual estimate ( $\mu\text{g/ml}$ )
30	29.961	0.197
45	45.080	0.195
60	59.954	0.338

The concentration of pentose (L(+) arabinose) in each solution was estimated 24 times. The standard deviations of the estimates do not differ from one another ( $P > 0.05$ ). No mean estimated concentration differs from its true value ( $P > 0.05$ ).



Table 3.11

The Estimation of Pentose: the Recovery of Pentose from a  
"Deproteinised" Solution

Concn. of arabinose in original soln. (mg/ml)	% recovery in "deproteinised" soln.
40	100.0
4	100.0
0.4	100.0
0.04	99.9

Duplicate 8 ml volumes were "deproteinised" with  
 $\text{ZnSO}_4/\text{NaOH}$  as described in Section 3.03.

coefficient of the system was about 5.5 ml/mg/cm. It did not depend on the pentose used, but did vary with the batch of p-bromoaniline, and tended to increase with time when a large number of estimates was made. Errors due to a change in extinction coefficient with time were reduced by estimating individual solutions in the symmetrical order which is discussed in Section 2.02.

#### 4.03 The Deproteinisation of a Sample

Samples were deproteinised by the  $\text{ZnSO}_4/\text{NaOH}$  procedure which is described in Section 3.03. Table 3.11 shows that there was no detectable difference between 100% and the mean percentage recovery of pentose in the supernatant of a deproteinised sample.

#### 4.04 The Precision of an Individual Estimate

The standard deviation of an individual estimate was found by repeatedly estimating a solution of known L(+) arabinose concentration. The results are presented in Table 3.12. Variance ratio tests suggested that the standard deviations of the estimated concentrations of the solutions did not differ from one another ( $P > 0.05$ ). Student's t-tests indicated that none of the mean estimated concentrations differed significantly from its true value ( $P > 0.05$ ), which supports the assumption of a linear relation between E and C.

#### 4.05 The Pentose Blank of a Haemolysate

The method for measuring a haemolysate's pentose blank was almost identical with that by which its inulin blank was evaluated (see Section 3.05). There were

Table 3.13

The Estimation of Pentose: the Pentose Blank of a  
Haemolysate

Mean volume of erythrocytes ( $v_o$ ml)	Mean pentose blank of haemolysate ( $H_p$ /ug)
0.9	6.2
1.8	7.1
2.7	7.4

The pentose blanks of four different donors' erythrocytes were measured as described in Section 4.05.

two differences: firstly, each ml of suspension medium contained 1 mg of L(+) arabinose and not 650  $\mu$ g of inulin; and secondly, the pentose concentration of the supernatants ( $C_p$   $\mu$ g/ml) was estimated. The pentose blank of a haemolysate ( $H_p$   $\mu$ g) was calculated from the equation:

$$H_p = (25 - (v_o - w_o)) \cdot C_p - 1000 \text{ } \mu\text{g.}$$

Table 3.13 gives the relationship between the mean values of both  $H_p$  and  $v_o$ , for four different donors' erythrocytes.  $H_p$  is small and variable. As the pentose content of the lower compartment of a special centrifuge tube was generally about 5 mg, the blank value of the cells in it, 2 to 3  $\mu$ g, was ignored.

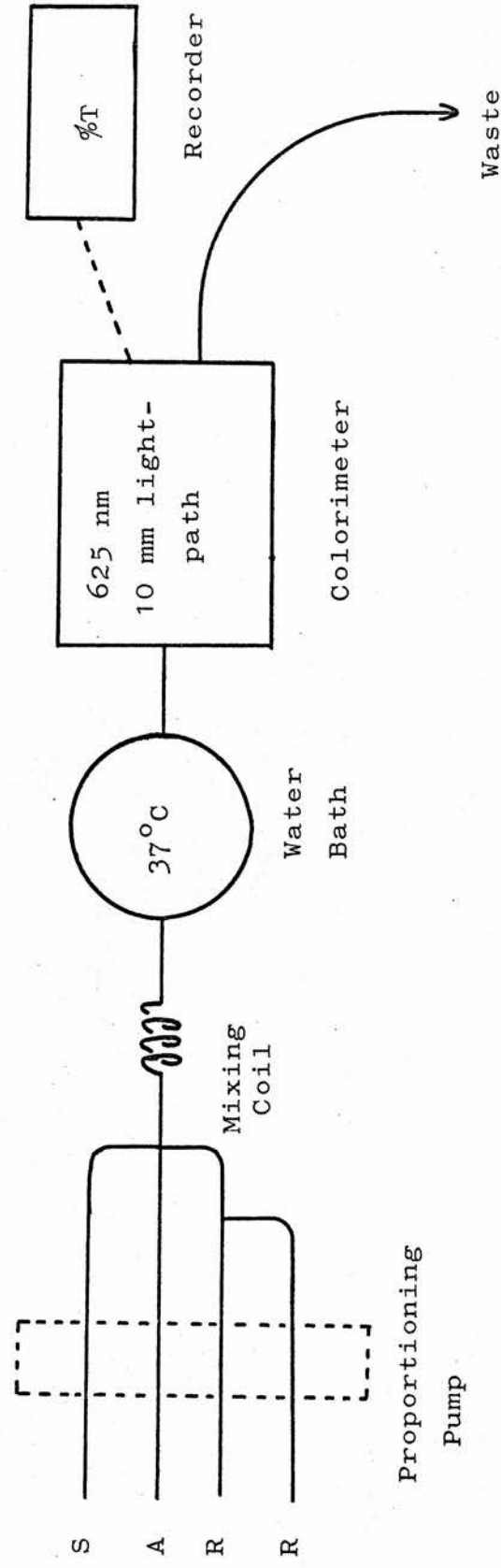
#### 4.06 The Interference of Inulin

The conclusion of Gilbert (1963), that the presence of inulin in no way alters the apparent concentration of pentose in a sample, was confirmed for a sample whose inulin to pentose concentration ratio, 20 to 45  $\mu$ g of inulin per  $\mu$ g of pentose, was greater than any encountered in practice. Four estimates were made of the pentose concentration of each of two samples, (1) and (2), each ml of which contained, respectively, 45  $\mu$ g of L(+) arabinose plus 20  $\mu$ g of inulin, and 45  $\mu$ g of L(+) arabinose. The means and the standard errors of the means of the estimates were:

sample (1): 45.02  $\pm$  0.03  $\mu$ g/ml;  
sample (2) 44.99  $\pm$  0.02  $\mu$ g/ml.

Figure 3.09

The Estimation of Glucose: the AutoAnalyser System



Flow rates: Sample (S): 2.0 ml/min      Sampling rate: 40/h  
 Air (A): 0.4 ml/min  
 Reagent (R): 2.0 + 2.0 = 4.0 ml/min      Reaction time = 5.3 min

The reagent is described in Section 5.02.



Section 5

The Estimation of Glucose

5.01 Introduction

The automated method for the estimation of glucose which was used in this work is one perfected by O'Brien (1969). It is based on a pair of coupled enzyme-catalysed reactions. D -D(+) glucose is first oxidised by molecular oxygen to D-glucono-5-lactone and hydrogen peroxide, in a reaction catalysed by D -D-glucose oxidase (D -D(+) glucose:  $O_2$  oxidoreductase, EC.1.1.3.4). Then peroxidase (donor:  $H_2O_2$  oxidoreductase, EC 1.11.1.7) catalyses the oxidation by hydrogen peroxide of a component of gum guaiacum resin to a coloured compound whose per cent transmittance at 625 nm is measured.

Figure 3.09 shows the arrangement of the AutoAnalyser system. The preparation of the reagent and some characteristics of the method are described below.

5.02 The Preparation of the Reagent

The reagent was prepared from the following:

- (i) Gum Guaiacum resin, supplied by either J.H.Bairds, Ltd., Edinburgh, or Brome & Schimmer, Ltd., London.
- (ii) Na acetate buffer, 1 M, pH 5.6.
- (iii) Triton X-100, supplied by Lenning Chemicals, Ltd., London: a 20% (v/v) solution in absolute ethanol, which is stable indefinitely at 4°C.
- (iv) Fermcozyme, from Hughes & Hughes, Ltd., Brentwood, Essex: the preparation contained 0.75 mg of glucose oxidase per ml, and was stable for at least 6 months at 4°C.

(v) Horse-radish peroxidase, from Hughes & Hughes, Ltd., Brentwood, Essex: a 0.1% (w/v) solution in distilled water, which was stable for 2 weeks at 4°C.

One litre of stock resin solution was made by extracting 0.75 g of finely-ground resin with 12.5 ml of solvent (95 vol. of ethanol: 5 vol. of water), filtering the extract into 600 ml of Na acetate buffer mixed with 20 ml of Triton X-100, and diluting the buffered extract to one litre with distilled water. Before use, the stock solution was kept at room temperature for a variable period which depended on the batch of resin, and was generally between one and seven days. Omission of this step diminished the reliability of the method.

One litre of reagent comprised 670 ml of stock resin solution, 10 ml of Fermcozyme, 10 ml of horse-radish peroxidase solution, and 310 ml of distilled water. The reagent was made up at least one day before it was required, because fresh reagent often gave a non-linear relationship between extinction and concentration.

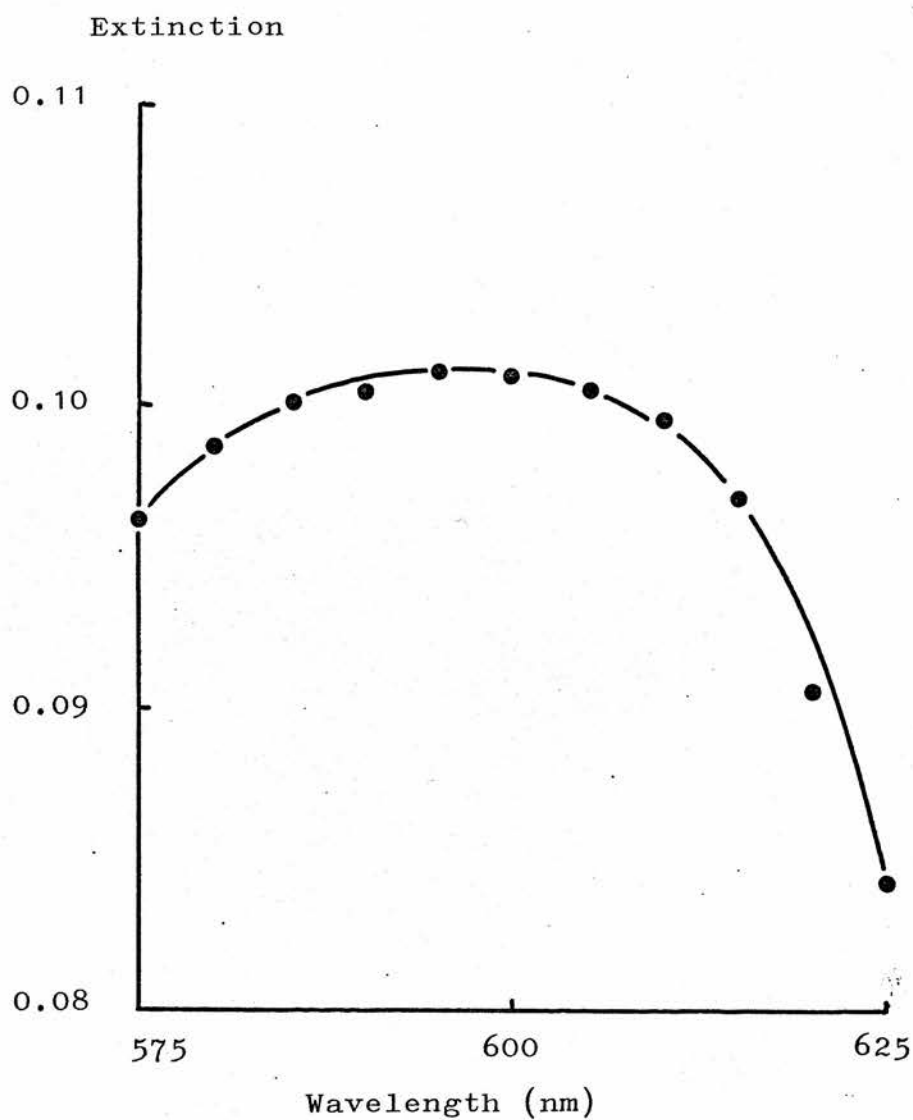
#### 5.03 The Absorption Spectrum of the Blue Product

The absorption spectrum of the coloured product formed from one batch of resin was determined between 575 nm and 625 nm. Since the coloured compound was unstable, the AutoAnalyser system was used for the determination.

As the colorimeter of an AutoAnalyser is supplied with a set of interference filters whose wavelengths

Figure 3.11

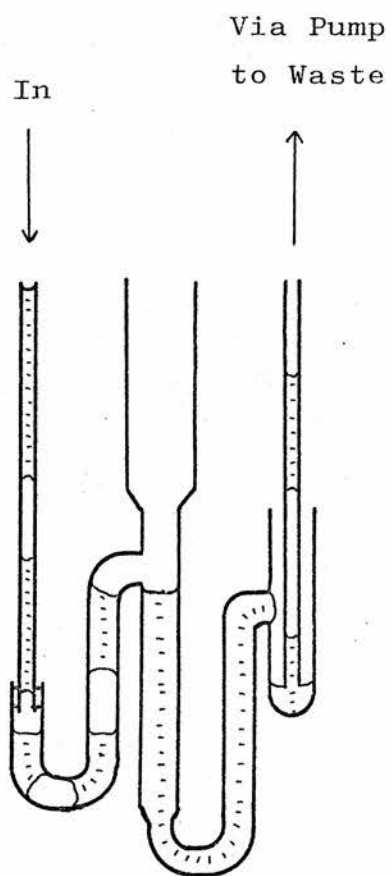
The Estimation of Glucose: the Absorption  
Spectrum of the Blue Product



The spectrum was determined in the adapted AutoAnalyser system described in Section 5.03.

Figure 3.10

The Technicon Flow-Cell, Modified for the Spectronic 20



differ by at least 30 nm, the unit is not suited for determining a spectrum. It was replaced by a "Spectronic 20 Colorimeter" (Bausch & Lomb, Rochester, New York, U.S.A.), which has a diffraction grating that generates a near-monochromatic light of any wavelength between 340 nm and 950 nm. A 6 mm Technicon flow-cell was modified in the Department expressly for use in the Spectronic 20 (it is depicted in Figure 3.10). The colorimeter was connected to a "VOM5 Recorder" (Bausch & Lomb), which registered the per cent transmittance of the flow-cell's contents. The sampling module shown in Figure 3.09 was replaced by a reservoir of sample (either water or a solution of 5  $\mu$ g glucose/ml).

At each wavelength, water was aspirated first. When the per cent transmittance had reached a constant value, it was adjusted to 100. The glucose solution was then aspirated; when the per cent transmittance again became constant, its value was noted.

Figure 3.11 is a plot of the spectrum. It is evident that the coloured product had an absorption maximum between 595 nm and 600 nm. Since the Auto-Analyser colorimeter did not have a filter whose wavelength lay between 550 nm and 625 nm, and since the extinction coefficient of the product at 625 nm was appreciably greater than that at 550 nm, per cent transmittance was measured at 625 nm.

#### 5.04 The Calibration of the System

The system was calibrated with three standard solutions, each ml of which contained either 4, 8 and 12,



Table 3.15

The Estimation of Glucose: a Relationship between Extinction  
and Concentration that is not of the First Order

True concn. of glucose ( $\mu\text{g/ml}$ )	Mean estimated concn. $\pm$ S.E.M. ( $\mu\text{g/ml}$ )	
	A	B
4	$4.05 \pm 0.01$	$4.00 \pm 0.01$
8	$7.90 \pm 0.02$	$7.98 \pm 0.02$
12	$12.05 \pm 0.01$	$12.01 \pm 0.02$

Each mean and its standard error (S.E.M.) were based on three estimates, which were derived from the following first-order regressions:

A : concentration (C) on extinction (E)

B :  $\log E$  on  $\log C$ .

Table 3.14

The Estimation of Glucose: the First-Order Relationship  
between Extinction and Concentration

True concn. of glucose ( $\mu\text{g/ml}$ )	Mean estimated concn. $\pm$ S.E.M. ( $\mu\text{g/ml}$ )
4	4.00 $\pm$ 0.01
8	7.99 $\pm$ 0.02
12	12.01 $\pm$ 0.02

Each mean and its standard error (S.E.M.) were based on three estimates derived from first-order regressions of concentration on extinction.

or 5, 10 and 15  $\mu\text{g}$  of an equilibrium mixture of a - and b -D(+) glucose. The precise relationship between extinction at 625 nm (E) and concentration (C) depended on the batch of resin used, and on the length of time for which the stock solution of resin had been kept. In most instances, of which the data in Table 3.14 represent one, E was taken to be linearly related to C, because the means of several successive estimates of the concentrations of the standard solutions, calculated from the first-order regression of C on E, did not differ appreciably from their true values. Occasionally E was clearly not linearly related to C, because the mean estimate of the concentration of each standard solution, calculated from this regression, differed markedly from its true value. Table 3.15 gives one example. In these instances, the concentration corresponding to a particular extinction was derived from the first-order regression of log E on log C. The Table also shows that when the estimates of the concentrations of the three standard solutions were derived in this way, their means were close to the true concentrations.

O'Brien (1969) showed that the per cent transmittance of a freshly-prepared solution of a -D(+) glucose decreased with time. This was due to the relatively slow rate at which glucose at room temperature attained mutarotational equilibrium. O'Brien therefore advised that a solution of glucose be heated in a bath of boiling water and then cooled to near room temperature before its concentration is estimated. His advice was invariably followed.

### 5.05 The Deproteinisation of a Sample

Deproteinisation of a sample with  $\text{ZnSO}_4/\text{NaOH}$  as described in Section 3.03 invariably caused the recovery of glucose to be incomplete. The inclusion of 1 g of the tri-sodium salt of diaminoethanetetra-acetic acid (EDTA) to 100 ml of the glucose oxidase reagent improved the recovery somewhat, though not to 100%. But when the amount of NaOH to be added was determined by titration to a phenolphthalein end-point, the recovery was 100%, even in the absence of EDTA. For example, each of three identical protein-free solutions, containing 100  $\mu\text{g}$  of glucose in 7 ml, was "deproteinised" by the addition of 1 ml of 10% (w/v)  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , and 2% (w/v) NaOH to a phenolphthalein end-point. The mixtures were made up to 10 ml with distilled water and centrifuged, and three estimates were made of the glucose concentration of each supernatant. The means, and the standard errors of the means, of the estimates of the glucose concentration of the supernatants were:

$9.98 \pm 0.02$ ,  $9.98 \pm 0.02$  and  $10.01 \pm 0.02$   $\mu\text{g}/\text{ml}$ .

It is clear that the means do not differ appreciably from one another or from their expected value of 10  $\mu\text{g}/\text{ml}$ , although, since the supernatants were not diluted before their concentration was estimated, the concentration of  $\text{Zn}^{2+}$  was maximal.

This method for deproteinisation has two disadvantages. Firstly, because the titre of NaOH is about 1.3ml, inulin is precipitated (see Section 3.03). Secondly, a haemolysate cannot be titrated to a

phenolphthalein end-point. Since the concentrations of both inulin and glucose in a haemolysate were usually required, the method was modified slightly. A known volume of haemolysate (either 8 or 10 ml) was treated with 1 ml of  $\text{ZnSO}_4$  and 1 ml of NaOH, and centrifuged. The inulin concentration of one aliquot of the supernatant was measured. Another aliquot was mixed with 0.25 ml of  $\text{ZnSO}_4$ , titrated with NaOH to a phenolphthalein end-point, diluted to 10 ml with distilled water, and centrifuged, to give a supernatant whose glucose concentration was estimated.

#### 5.06 The Precision of an Individual Estimate

O'Brien (1969) derived the standard deviation of an individual estimate by estimating several times a solution of known glucose concentration, using reagent prepared from different batches of resin. He concluded that the standard deviation was 0.06  $\mu\text{g}$  glucose /ml, for all concentrations between 5 and 15  $\mu\text{g}$  glucose /ml.

#### 5.07 The Glucose Blank of a Haemolysate

The method for the determination of the glucose blank of a haemolysate was similar to that described in Section 3.05 for the determination of an inulin blank. A solution containing 200  $\mu\text{g}$  of glucose was added to a known volume of cells, which were immediately haemolysed by the addition of distilled water. The haemolysate was deproteinised at once (to prevent metabolism of glucose), and its glucose concentration was estimated.



Table 3.17

The Estimation of Glucose: the Interference of Inulin

True concn. of glucose in sample ( $\mu\text{g/ml}$ )	Concn. of inulin in sample ( $\mu\text{g/ml}$ )	Mean estimated concn. of glucose in sample ( $\mu\text{g/ml}$ )
10	1	10.00
10	10	9.98
10	100	10.02

The concentration of glucose in each sample was estimated twice.

Table 3.16

The Estimation of Glucose: the Glucose Blank of a  
Haemolysate

Mean volume of erythrocytes (ml)	Mean glucose blank of haemolysate ( $\mu$ g)
0.3	- 2.8
0.6	1.2
0.9	0.3

The glucose blank of two different preparations of washed erythrocytes was determined as described in Section 5.07.

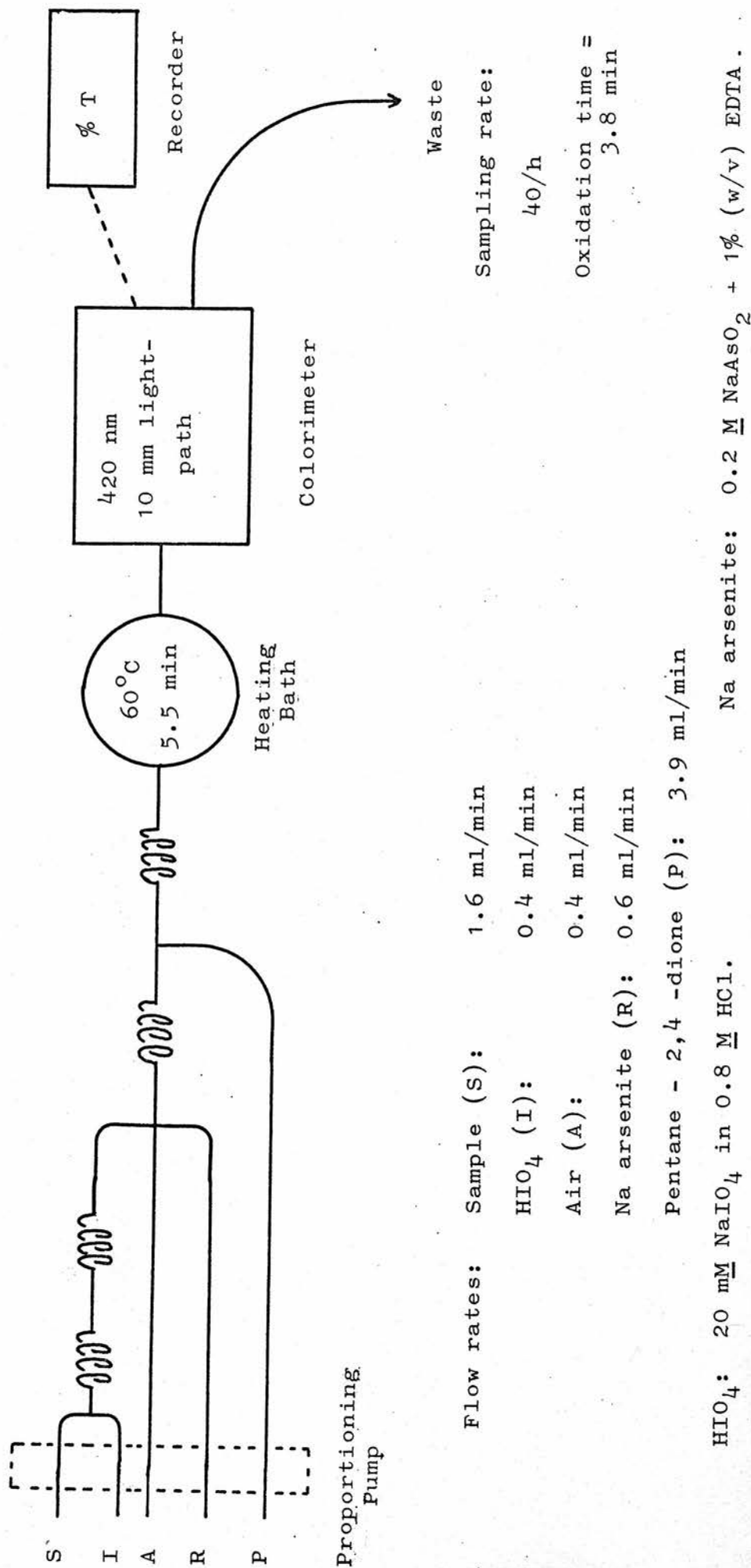
Table 3.16 shows that the washed erythrocytes contained nothing that estimated as glucose. In consequence, it was concluded that the erythrocytes were initially free of glucose (see p.99 ).

#### 5.08 The Interference of Inulin

The apparent glucose concentration of samples of known glucose and inulin concentrations were measured. The results are presented in Table 3.17. It is evident that even when the ratio of the amount of inulin to that of glucose was 10  $\mu\text{g}$  inulin /  $\mu\text{g}$  glucose (which is a greater value than any encountered in practice), there was no appreciable difference between an apparent and a true concentration. Therefore the presence of inulin does not interfere with this method for estimating glucose.

Figure 3.12

The Estimation of Erythritol: the AutoAnalyser System



## Section 6      The Estimation of Erythritol

### 6.01    Introduction

Ryley (1955) described a method that can be used to estimate any polyhydric alcohol with a  $-\text{CHOH}-\text{CH}_2\text{OH}$  group. The alcohol is first oxidised by periodic acid to formaldehyde. Excess periodic acid is then reduced by Na arsenite. Finally, the formaldehyde is heated at  $100^\circ\text{C}$  with chromotropic acid to give a product which absorbs at 580 nm. This method could have been automated, even though extreme conditions of pH and temperature are required to generate the coloured product. However, formaldehyde also reacts with pentane - 2, 4 - dione, under much milder conditions, to give a complex absorbing at 412 nm (Nash, 1953). The automated method eventually developed was based on the latter colorimetric reaction.

### 6.02    The AutoAnalyser System

Figure 3.12 depicts the AutoAnalyser system. The sample and periodic acid were combined in a capillary T-piece. They took 4 min to pass through the delay coil, which was at room temperature.

The periodic acid reagent was prepared just before use by mixing 10 vol. of  $0.1\text{M}$   $\text{NaIO}_4$  with 40 vol. of  $\text{M}$   $\text{HCl}$ . Low pH was chosen to minimise oxidation of hexoses (Fleury, Courtois & Bieder, 1952).



Table 3.19

The Estimation of Erythritol: the Precision of an Estimate

True concn. of erythritol (nmol/ml)	Mean estimated concn.of erythritol (nmol/ml)	Standard deviation of an individual estimate (nmol/ml)
25	25.5	0.7
50	48.9	1.2
75	75.4	1.6

The concentration of erythritol in each  
solution was estimated 6 times.

Table 3.18

The Estimation of Erythritol: the Recovery of Erythritol  
from a "Deproteinised" Solution

Concn. of erythritol in original soln. ( $\mu\text{mol/ml}$ )	% recovery in "deproteinised" soln.
12.5	102.0
1.25	97.6
0.125	101.2

Duplicate 8 ml volume of protein-free solution were "deproteinised" by the addition of 1 ml of 10% (w/v)  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  followed by 1 ml of 2% (w/v) NaOH, as described in Section 3.03.

### 6.03 The Calibration of the System

The system was calibrated with three standard solutions, containing 25, 50 and 75 nmol erythritol/ml. Extinction at 420 nm (E) was assumed to be proportional to concentration (C) because, in different experiments,  $d^2E/dC^2$  was either positive or negative, and never far from zero. The extinction coefficient was about 3.4 ml/ $\mu$ mol erythritol./cm.

### 6.04 The Deproteinisation of a Sample

When the Na arsenite contained no EDTA, the recovery of erythritol in the supernatant of a sample deproteinised by the  $ZnSO_4/NaOH$  procedure given in Section 3.03 was always significantly less than 100%. However, the data in Table 3.18 show that when 10 mg of EDTA were included in each ml of the Na arsenite solution, recoveries of about 100% were observed.

### 6.05 The Precision of an Individual Estimate

The standard deviation of an individual estimate was found by repeatedly estimating solutions of known erythritol concentration. The results are summarised in Table 3.19. It is evident that the method lacks precision, because the coefficients of variation of the estimates are between 2% and 3%.

There are three obvious reasons for the lack of precision. Firstly, the mixing at the capillary T-piece of sample and periodic acid may have been incomplete. Secondly, this mixture was not segmented by

Table 3.20

The Estimation of Erythritol: the Erythritol Blank of a  
Haemolysate

Volume of erythrocytes ( $v_o$ ml)	Mean erythritol blank of haemolysate ( $H_e$ nmol)
0.8	38.3
1.6	52.0
2.4	99.6

One micromole of erythritol was added to the cells.  
Each blank is the mean of two determinations on  
erythrocytes from one donor.

The first-order regression of  $H_e$  on  $v_o$  is:

$$H_e = 39.1 (\pm 5.5) \cdot v_o + 0.6 (\pm 8.2)$$

bubbles of gas as it spiralled through the delay coil. Hence an appreciable interaction between successive samples could have occurred. Lastly, the rates of flow of the reactants differed markedly, and therefore tended to be pulsatile rather than uniform.

The choice of the rates of flow is limited by several constraints, of which two deserve comment. The first is that sufficient Na arsenite must be added to reduce all the excess periodic acid to iodide and not just to free iodine. And the second is that Na arsenite is sparingly soluble, especially at low pHs. (The faint cloudiness that was observed in the mixture of sample, periodic acid and Na arsenite was attributed to precipitation of the arsenite).

#### 6.06 The Erythritol Blank of a Haemolysate

The erythritol blank of a haemolysate ( $H_e$  nmol) was determined in a manner like that given for inulin in Section 3.05. Table 3.20 is a summary of the values that were found for one donor's erythrocytes (of volume  $v_o$  ml). The first-order regression of  $H_e$  on  $v_o$  is:

$$H_e = 39.1 (\pm 5.5) \cdot v_o + 0.6 (\pm 8.2)$$

Therefore the blank was taken as being 39.1 nmol of erythritol per ml of cells.

#### 6.07 The Interference of Inulin

The apparent concentrations of erythritol in solutions containing both this compound and inulin were estimated. The estimates are given in Table 3.21.



Table 3.21

The Estimation of Erythritol: the Interference of Inulin

Erythritol concn. of sample (nmol/ml)	Increase in apparent erythritol concn.(nmol/ml) when the sample contained inulin of concn:		
	0.2 /ug/ml	0.6 /ug/ml	1.0 /ug/ml
25	0.5	0.5	0.7
50	0.1	0.8	0.7
75	0.1	0.3	1.0

Each erythritol concentration was estimated twice.

The first-order regression of the increase in apparent erythritol concentration,  $Z_e$  nmol/ml, on inulin concentration,  $C_i$  /ug/ml, is:

$$Z_e = 0.7(\pm 0.2) \cdot C_i + 0.1(\pm 0.1)$$

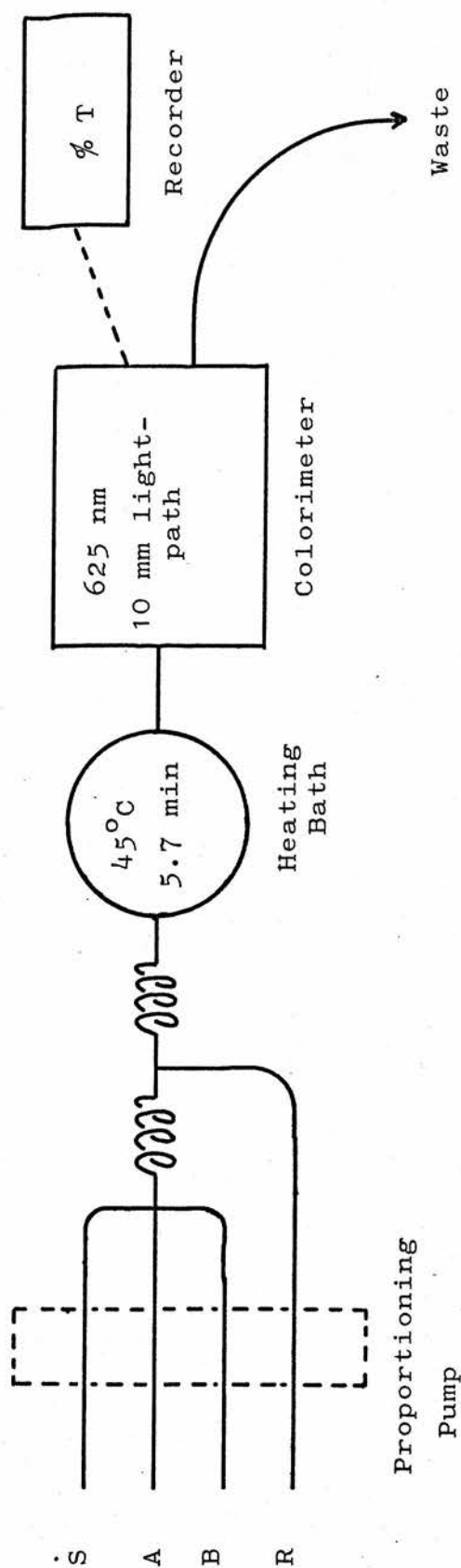
The relationship of the apparent increase in erythritol concentration,  $Z_e$  nmol/ml, to inulin concentration,  $C_i$  /ug/ml, does not seem to depend on erythritol concentration, and is summarised by the first-order regression:

$$Z_e = 0.7 (\pm 0.2) \cdot C_i + 0.1 (\pm 0.1)$$

Hence the apparent concentration of erythritol in a solution also containing inulin was "corrected" by the subtraction of  $(0.7 \cdot C_i)$  nmol/ml. In practice a correction did not exceed 5%. Its small magnitude supports the assertion of Fleury et al. (1952), that at acid pHs periodic acid does not liberate much formaldehyde from fructose.

Figure 3.13

The Estimation of Thiourea: the AutoAnalyser System



Flow rates: Sample (S): 1.6 ml/min  
Air (A): 0.6 ml/min  
Buffer (B): 2.9 ml/min  
Reagent (R): 1.6 ml/min

Sampling rate: 40/h

Buffer: 0.1 M Na citrate - citric acid, pH 3.8, with 5 g of EDTA per litre.

Reagent: 1:10 dilution of stock Grote's reagent.

## Section 7

## The Estimation of Thiourea

### 7.01 Manual Method

A manual method for the estimation of thiourea in orange juice was described by Anon. (1948). Its basis is the reaction of thiourea with Grote's reagent to form a blue compound (Grote, 1931). 10 ml of 0.6% (w/v) Na citrate-acetic acid buffer, pH 3.5-5.5, containing from 50 to 150  $\mu$ g of thiourea, are mixed with 1 ml of reagent, and incubated for either 45 min at 20 - 25°C, or 15 min at 45°C. The extinction of the mixture at 610 nm is linearly related to the concentration of thiourea. The method can be adapted for use on the AutoAnalyser because:

- (i) it is colorimetric;
- (ii) the reagents do not attack Tygon pump tubing;
- (iii) the reactants are incubated together for a short time at a moderate temperature;
- (iv) the method is sensitive.

### 7.02 The AutoAnalyser System

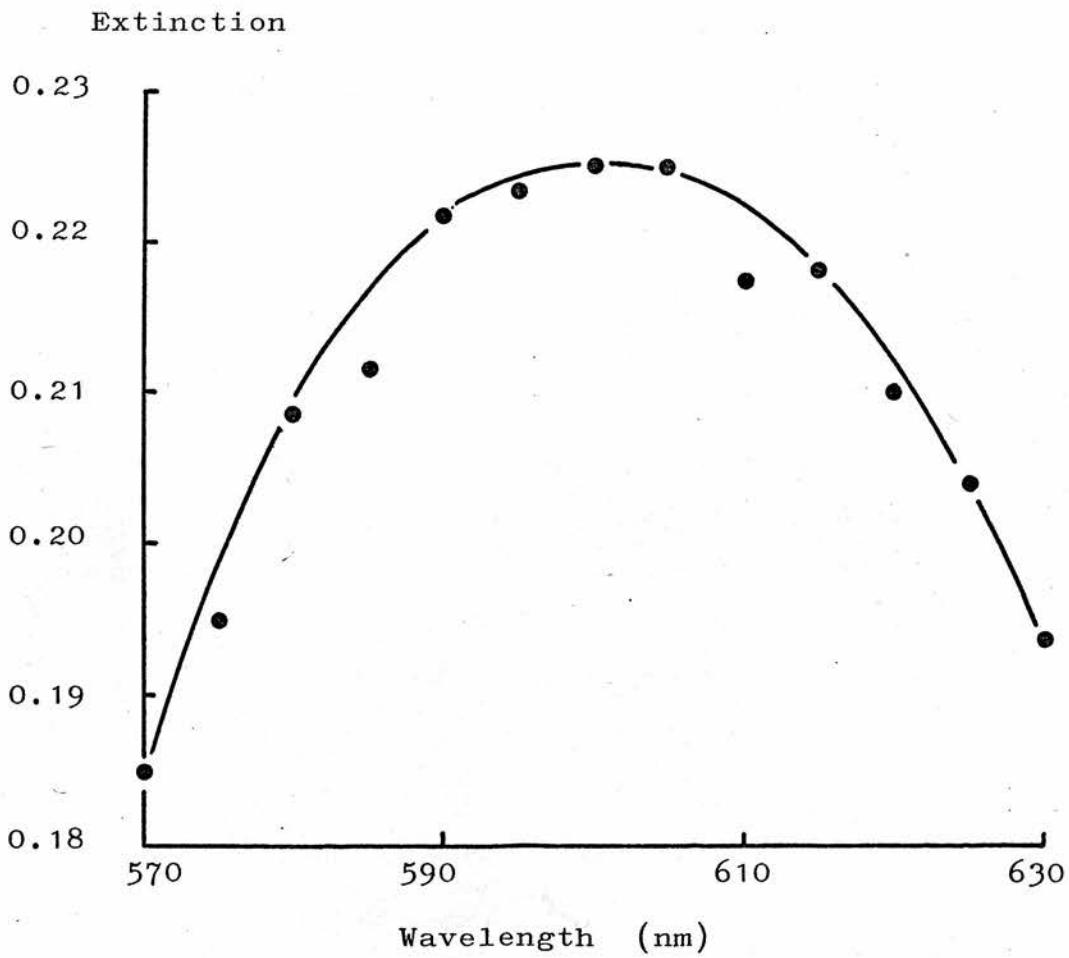
The AutoAnalyser system is shown in Figure 3.13. The reagent was prepared just before use by diluting 1 vol. of stock Grote's reagent with 9 vol. of distilled water. The buffer, which contained 5 g of EDTA per litre, was 0.1 M Na citrate-citric acid, pH 3.8.

### 7.03 The Preparation of the Stock Reagent

The stock reagent was prepared as follows. To a solution of 5 g of Na nitroprusside in 100 ml of distilled water were added 5 g of hydroxylamine hydrochloride,

Figure 3.14

The Estimation of Thiourea: the Absorption  
Spectrum of the Blue Product



The determination of the spectrum is described  
in Section 7.04.

followed immediately by 10 g of  $\text{NaHCO}_3$ . The whole was allowed to stand until most of the carbon dioxide had been evolved, whereupon 1 ml of bromine was added. The mixture was shaken until there was no further effervescence, and was then filtered. The filtrate was diluted to 250 ml with distilled water, and allowed to stand at room temperature for 5 to 10 h. It was stable for several weeks when stored in the dark at  $4^\circ\text{C}$ .

Roughly one in every two batches of the stock reagent failed to react appreciably with thiourea. This lack of reactivity was never explained.

#### 7.04 The Absorption Spectrum of the Blue Product

The absorption spectrum of the blue product was determined between 570 nm and 630 nm in an AutoAnalyser, in a manner similar to that described in Section 5.03. Distilled water, and then a solution containing 30  $\mu\text{g}$  thiourea/ml, were aspirated at each wavelength.

The spectrum, which is plotted in Figure 3.14, has a maximum between 600 nm and 605 nm. The per cent transmittance of the reaction mixture was routinely measured at 625 nm.

#### 7.05 The Effect of Temperature

When the reaction mixture took 5 min 45 s to pass through the heating bath, a bath temperature of  $45^\circ\text{C}$  resulted in a maximum in the extinction coefficient. This maximum value was unaffected by a change in the pH of the 0.1 M Na citrate-citric acid buffer from 3.5 to 4.5,



Table 3.22

The Estimation of Thiourea: the Effect of Reagent  
Concentration on Extinction Coefficient

Dilution of stock reagent	Extinction coefficient (ml/ $\mu$ g thiourea/cm)	Reagent blank (units)
1:4	0.0075	0.100
1:10	0.0073	0.025
1:20	0.0052	0.011

The measurement of the extinction coefficient is described in Section 7.06.

or from 4.5 to 5.5. Routinely, the heating bath was at a temperature of  $45^{\circ}\text{C}$ , and the buffer was of pH 3.8.

#### 7.06 The Effect of Reagent Concentration

Table 3.22 gives the extinction coefficients (for samples containing between 10 and 40  $\mu\text{g}$  thiourea/ml), and the reagent blanks (that is, the extinctions of samples of distilled water), obtained by diluting 1 vol. of stock Grote's reagent with distilled water to the stated final volumes. The 1:10 dilution of stock reagent gave nearly as large an extinction coefficient as, and a much smaller reagent blank than, the 1:4 dilution. Therefore it was used routinely.

#### 7.07 The Calibration of the System

The system was usually calibrated with three standard solutions containing 10, 20 and 30  $\mu\text{g}$  thiourea/ml. Extinction at 625 nm (E) was assumed to be linearly related to concentration (C) since, in different experiments  $d^2E/dC^2$  was either positive or negative, and always close to zero.

#### 7.08 The Deproteinisation of a Sample

When the 0.1 M citrate buffer contained no EDTA, the presence of 5 mequiv. of  $\text{Zn}^{2+}$  per litre of sample interfered with an estimation by causing the record of per cent transmittance against time to be "spiky", and the extinction coefficient of the method to decrease. The inclusion of 5 g of EDTA per litre of buffer abolished the interference without appearing to affect the

Table 3.26

The Estimation of Thiourea: the Interference of Inulin

Thiourea concn. of sample ( $\mu\text{g/ml}$ )	Increase in apparent thiourea concn. ( $\mu\text{g/ml}$ ) when the sample contained inulin of concn.:		
	10 $\mu\text{g/ml}$	20 $\mu\text{g/ml}$	30 $\mu\text{g/ml}$
10	0.03	0.07	0.03
20	- 0.12	0.05	0.09
30	0.26	0.23	0.12

Each thiourea concentration was estimated twice.

Table 3.25

The Estimation of Thiourea: the Thiourea Blank of a  
Haemolysate

Volume of erythrocytes (ml)	Mean thiourea blank of haemolysate ( $\mu$ g)
1.2	- 1.2
2.4	3.0
3.6	9.0

Three milligrams of thiourea were added to the cells.  
Each blank is the mean of two determinations on  
erythrocytes from one donor.

Table 3.24

The Estimation of Thiourea: the Precision of an Estimate

True concn. of thiourea ( $\mu\text{g/ml}$ )	Mean estimated concn. of thiourea ( $\mu\text{g/ml}$ )	Standard deviation of an individual estimate ( $\mu\text{g/ml}$ )
10	9.98	0.03
20	20.03	0.05
30	29.99	0.06

The concentration of thiourea in each solution was estimated 7 times.

The means of the estimates do not differ from the true values ( $P > 0.05$ ).

The standard deviations do not differ ( $P > 0.05$ ).

Table 3.23

The Estimation of Thiourea: the Recovery of Thiourea  
from a "Deproteinised" Solution

Concn. of thiourea in original soln. (mg/ml)	% recovery in "deproteinised" soln.
1.25	99.8
0.125	99.6
0.0125	102.0

Duplicate 8 ml volumes were "deproteinised" with  $\text{ZnSO}_4/\text{NaOH}$  as described in Section 7.08.



method in any other way.

Samples were deproteinised by the  $\text{ZnSO}_4/\text{NaOH}$  procedure described in Section 3.03. Table 3.23 demonstrates that when duplicate 8 ml volumes of protein-free solutions, containing either 0.1, 1 or 10 mg of thiourea, were each deproteinised in this way, virtually 100% of the thiourea was recovered in the supernatant.

#### 7.09 The Precision of an Individual Estimate

The standard deviation of an individual estimate was found by repeatedly estimating solutions of known thiourea concentration. The results are presented in Table 3.24. The standard deviations do not differ from one another (variance ratio test;  $P > 0.05$ ), and the mean estimated concentrations are not different from their true values (Student's t-test;  $P > 0.05$ ).

#### 7.10 The Thiourea Blank of a Haemolysate

The thiourea blank of a haemolysate was determined in a manner similar to that given for inulin in Section 3.05. The data in Table 3.25 suggest that the value for the blank is zero.

#### 7.11 The Interference of Inulin

The data in Table 3.26 demonstrate that inulin, when present at a concentration of up to 30  $\mu\text{g/ml}$ , did not affect the estimation of thiourea.

## Chapter XIII

### SUMMARY

1. The preparation and some of the properties of a suspension of washed erythrocytes are given. Although the cells differ from those in vivo, their properties are stable. Inulin appears to be a reliable marker of extra-erythrocyte water. (Chapter X).
2. The design of the waisted centrifuge tubes and plungers is discussed. They can be used to determine a time-course of permeation provided that its half-time is at least 1 min. The accuracy of such a determination depends on the homogeneity of the extra-cellular media in the two compartments at the instant when, but not after, a tube is divided. Precise determinations require that the methods employed to estimate permeant and inulin be of high precision. (Chapter XI).
3. These estimates were made on an AutoAnalyser, in a manner designed to enhance their reliability. Methods are given for the estimation of inulin, pentoses, D(+) glucose, erythritol and thiourea. With the possible exception of the method for erythritol, they meet the above requirement. (Chapter XII).
4. The procedure used to determine a typical time-course is described. (Chapter VIII).

PART   FOUR

RESULTS

In this Part, the principal experimental results of the investigations are presented and discussed. They are grouped into three Chapters, whose contents are summarised below.

Chapter XV is devoted to the permeation of the three aldoses (D(+) glucose, L(+) arabinose and D(+) xylose). Since the theory in Part Two can only be applied to those non-electrolytes which at equilibrium are uniformly distributed through the intracellular and extracellular water, it is first shown that both pentoses, neither of which is metabolised by the human erythrocyte, appear to satisfy this condition. It is assumed that glucose, which is metabolised by the erythrocyte, also satisfies the condition.

A measure of the relative permeability of the erythrocyte to a - and b - D(+) glucose is then given. This demonstrates that the two anomers penetrated the cell at different rates.

Next it is shown that the time-courses of permeation of the aldoses conform to simple carrier kinetics and are inconsistent with any diffusion hypothesis. Whereas the transfer of each aldose is characterised by a unique value of K, the three estimates of V are of the same order of magnitude. However, in view of the distinctive properties of a - and b - glucose, the parameters may be composite.

The effect of temperature on the composite parameters is reported. It seems that as temperature increased the values of K remained constant, whereas all the values of V increased markedly in an identical manner. The latter observation supports the suggestion that the aldoses share a common carrier, and that it is the translocation of this carrier across the membrane which limits their rate of permeation. It also permits the calculation of the energy of activation required for translocation.

The last experiment recounted in this Chapter is concerned with the effect of insulin on the permeation of glucose. The permeability of the erythrocyte is alleged to be insensitive to added hormone. So the cells were incubated with anti-insulin serum before their permeability was measured. The results of the experiment were indecisive.

The Chapter concludes with a discussion. The results obtained in this investigation are compared with those of other workers, and their physical meaning is touched upon.

Chapters XVI and XVII describe the permeation of meso-erythritol and thiourea, respectively. The first of these compounds, like the aldoses, appears to enter the erythrocyte by a system of facilitated diffusion. Its characteristics are presented. The second seems to be concentrated within the erythrocyte. This prevents an analysis of its permeation.

The final Chapter in the Part is a Summary.

Table 4.01

The Distribution of the Pentoses at Equilibrium

Pentose	Mean concn. ± S.E.M. (mM)	10 <sup>2</sup> .mean f' ± S.E.M.	Mean recovery ± S.E.M. (%)	
			pentose	inulin
L(+) arabinose	123.1 ± 0.1	100.2 ± 0.3	100.5 ± 0.2	99.9 ± 0.1
	25.6 ± 0.0	100.3 ± 0.3	99.7 ± 0.3	100.2 ± 0.3
D(+) xylose	124.2 ± 0.2	100.8 ± 0.5	98.9 ± 0.5	99.5 ± 0.3
	25.5 ± 0.1	99.9 ± 0.4	99.6 ± 0.1	100.0 ± 0.1

Each mean is based on 4 different experiments.

The erythrocytes were incubated with pentose for 8 h at 23.5°C.



Chapter XV THE PERMEATION OF MONOSACCHARIDES

Section 1 The Distribution of the Pentoses at Equilibrium

The equilibrium distribution of the pentoses between intra- and extra- erythrocyte water was investigated as follows. To 5 ml of a suspension of washed erythrocytes, of haematocrit 40%, were added 2 ml of an incubation medium that contained a total of either 0.6 nmol or 0.1 nmol of pentose. The mixture was shaken mechanically for 8 h at 23.5 °C. Then it was centrifuged at 3,000 x g for 10 min, and all the supernatant was removed. The concentrations of pentose and inulin in both the supernatant and the mass of packed cells were estimated. The total volume of water in 5 ml of the 40% suspension was found by drying aliquots to constant weight, as described in Part Three, Chapter X. The concentration of intracellular pentose after the 8 h incubation was expressed as the fraction  $f'$ .

The results are in Table 4.01. They corroborate the fundamental postulates that all the intracellular water is available to the pentoses, and that these pentoses are not metabolised by the human erythrocyte (Lachhein & Matthies, 1960). Alternatively, if the postulates are accepted, the results suggest that the estimate of the content of intracellular water is accurate (see Part Three, Chapter X).

The equilibrium distribution of D(+) glucose was not measured accurately, because this monosaccharide

is metabolised by the human erythrocyte at a rate of between 1.5 and 2.0 mmol/1 cells/hr (Whittam, 1964). However, there are two reasons for supposing that all the water in an erythrocyte can act as a solvent for glucose. The first is that  $f'$  approached unity as a time-course was prolonged. For example, when erythrocytes had been incubated in 23.1 mM glucose at 24.3°C for 25 min, the mean of two values of  $f'$  was 0.96. The second is that Gary Bobo (1967) inferred that glucose is distributed throughout the water of a concentrated solution of haemoglobin. Therefore the analysis in Part Two can be applied to time-courses of permeation of all three aldoses.

Table 4.02

The Permeation of Glucose: the Precision of  $f'$ 

Concn. of glucose (mM)	Time (min)	Mean $f'$ $\pm$ S.D.	Coefft. of varn. of $f'$ (%)
46.4	1.40	$0.380 \pm 0.014$	3.7
	5.00	$0.721 \pm 0.025$	3.5
23.1	0.40	$0.321 \pm 0.046$	14.3
11.6	0.75	$0.690 \pm 0.055$	8.0

Each mean is based on 4 determinations.

Temperature =  $23.5^{\circ}\text{C}$ .

Section 2

The Precision of an Estimate of  $f'$

The precision of an individual estimate of  $f'$  was determined for glucose, by making four independent estimates of this variable at a fixed time. The data are summarised in Table 4.02. They demonstrate that the coefficient of variation of  $f'$  was low provided that  $t$  was greater than about 1 min. On the other hand, reliable estimates were not obtained when the period of incubation was short. This limitation on the use of the waisted centrifuge tubes is discussed in Chapter XI of Part Three. It precluded the investigation of the permeation of an aldose at a temperature greater than about  $30^{\circ}\text{C}$ , or at a concentration of extracellular aldose that, as it transpired, was not appreciably greater than  $K$ .

Table 4.03

The Permeation of "a-", "Mutarotated", and "b-" (D(+)) Glucose

Concn. of glucose (mM)	Time (min)	"a-"	Mean f' $\pm$ S.E.M. "m-"	"b-"
54.1	1.5	0.285 $\pm$ 0.006	0.318 $\pm$ 0.007	0.353 $\pm$ 0.005
	5.0	0.664 $\pm$ 0.008	0.672 $\pm$ 0.008	0.691 $\pm$ 0.005
24.6	1.0	0.316 $\pm$ 0.007	0.340 $\pm$ 0.010	0.386 $\pm$ 0.007
	3.5	0.748 $\pm$ 0.011	0.752 $\pm$ 0.017	0.764 $\pm$ 0.012

"a-", "m-" and "b-" refer to a-, mutarotated, and b-  
D(+) glucose.

Each mean is based on 4 determinations.

Temperature = 23.0 - 23.8°C.

Section 3     The Permeation of  $\alpha$  - and  $\beta$  - D(+) Glucose

The stereo-specificity of the mechanism of permeation of D(+) glucose was investigated as follows. Erythrocytes were incubated with a medium containing a fixed concentration of either " $\alpha$  -", "mutarotated", or " $\beta$  -" D(+) glucose, and  $f'$  was measured. Since both anomers mutarotate in aqueous solution, an incubation medium that contained only one anomer was prepared 30 s before "time zero" by dissolving the anomer in volume medium. The medium of "mutarotated" glucose was made from  $\alpha$  - glucose, and warmed for 10 min at 70°C.

Table 4.03 shows the results. They suggest that in the conditions used the relative rates of permeation are:  $\beta$  - glucose > mutarotated glucose >  $\alpha$  - glucose. Hence the rate-limiting mechanism appears to distinguish between the two anomers of glucose.

A detailed study of the difference in permeability was not attempted, for two reasons. Firstly, as already mentioned, both anomers mutarotate in aqueous solution, the half-time for the reaction being 7.5 min at a pH of 7.1 and a temperature of 24°C (Keston, 1963). Secondly, erythrocytes have an enzyme, "mutarotase", that catalyses the mutarotation of glucose (Sacks, 1968). Since there are no data on the activity of this enzyme per unit volume of erythrocytes (W. Sacks, personal communication), the relative concentrations of the two anomers within the erythrocytes are unknown.



## Section 4 Time-Courses of Permeation at Different Concentrations

### 4.01 Theory

This sub-section is a brief resumé of the way in which the three permeation equations were fitted to an experimental time-course, and the goodness of the fits assessed.

#### (1) The Simple Diffusion and the Simple Membrane Carrier Hypotheses.

- (i) Both hypotheses predict that the relationship between  $F$  and  $L$  is linear. This prediction was tested by inspecting a plot of  $F$  against  $L$ ; and by comparing with the known variability of  $f'$  the coefficient of variation of  $f'$ ,  $c(f')$ , estimated from the residual variance about the unweighted first-order regression of  $F$  on  $L$ ,  $s^2$ , and the number of observations,  $n$ :

$$c(f') = \sqrt{\frac{n \cdot s^2}{\sum F^2}}$$

The two hypotheses were then distinguished on a quantitative basis:

- (ii) The coefficients of two unweighted first-order regressions relating  $F$  and  $L$  were calculated:

$$F = b' \cdot L + a'$$

$$L = b'' \cdot F + a''$$

The standard errors of  $b'$  and  $a'$ ,  $s(b')$  and  $s(a')$ , were also found.

According to the Simple Diffusion Hypothesis,  $b'$  should be equal to  $(m+x)/x$ , and  $b''$  should be equal to  $x/(m+x)$ . Conversely, on the Simple Membrane Carrier Hypothesis:

$$(m+x)/x > b' > 1$$

$$\text{and } x/(m+x) < b'' < 1$$

so that  $K$  and  $V$  are finite and positive.

- (iii) The best-fit estimates of the appropriate characteristic parameters were derived:

$$\text{either } P.A = \frac{1}{2} \left( -\frac{x}{m} \cdot a' + \frac{m+x}{m} \cdot a'' \right) ;$$

$$\text{or } K = \frac{1}{2} (K' + K'')$$

$$\text{where } K' = \frac{(b'-1) \cdot x \cdot (m+x)}{m - (b'-1) \cdot x} \text{ and } K'' = \frac{(1-b'') \cdot x \cdot (m+x)}{m \cdot b'' - (1-b'') \cdot x}$$

$$\text{and } V = \frac{1}{2} (V' + V'')$$

$$\text{where } V' = \frac{-a' \cdot (K'+m+x) \cdot (K'+x) \cdot x}{K' \cdot m} \text{ and}$$

$$V'' = \frac{a''}{b''} \cdot \frac{(K''+m+x) \cdot (K''+x) \cdot x}{K'' \cdot m}$$

The standard errors of K and V,  $s(K)$  and  $s(V)$ , were found from  $s(b')$  and  $s(a')$ .

(2) The Bulk Diffusion Hypothesis.

- (i) The analysis was only carried out when the permeant contributed less than 15% of the total extracellular osmolality.
- (ii) z was calculated from  $f'$ , and plotted against t. The plot should be linear.
- (iii) The unweighted first-order regression of z on t was calculated:

$$z = p \cdot t + q$$

q should not differ significantly from zero (test: Student's t-test). The mean coefficient of variation of  $f'$ ,  $c(f')$  estimated from the residual variance about the regression, should be of the same order of magnitude as that determined experimentally.

- (iv) The second-order regression of z on t was calculated. The addition of the term in  $t^2$  should not improve the fit of the regression to the data (test: analysis of variance).
- (v) If all three conditions were satisfied, the time-course was said to be consistent with the Bulk Diffusion Hypothesis. The best-fit estimate of  $D/e^2$  was equated to  $p_0$ , the coefficient of the first-order regression  $z = p_0 \cdot t$ .

Table 4.05

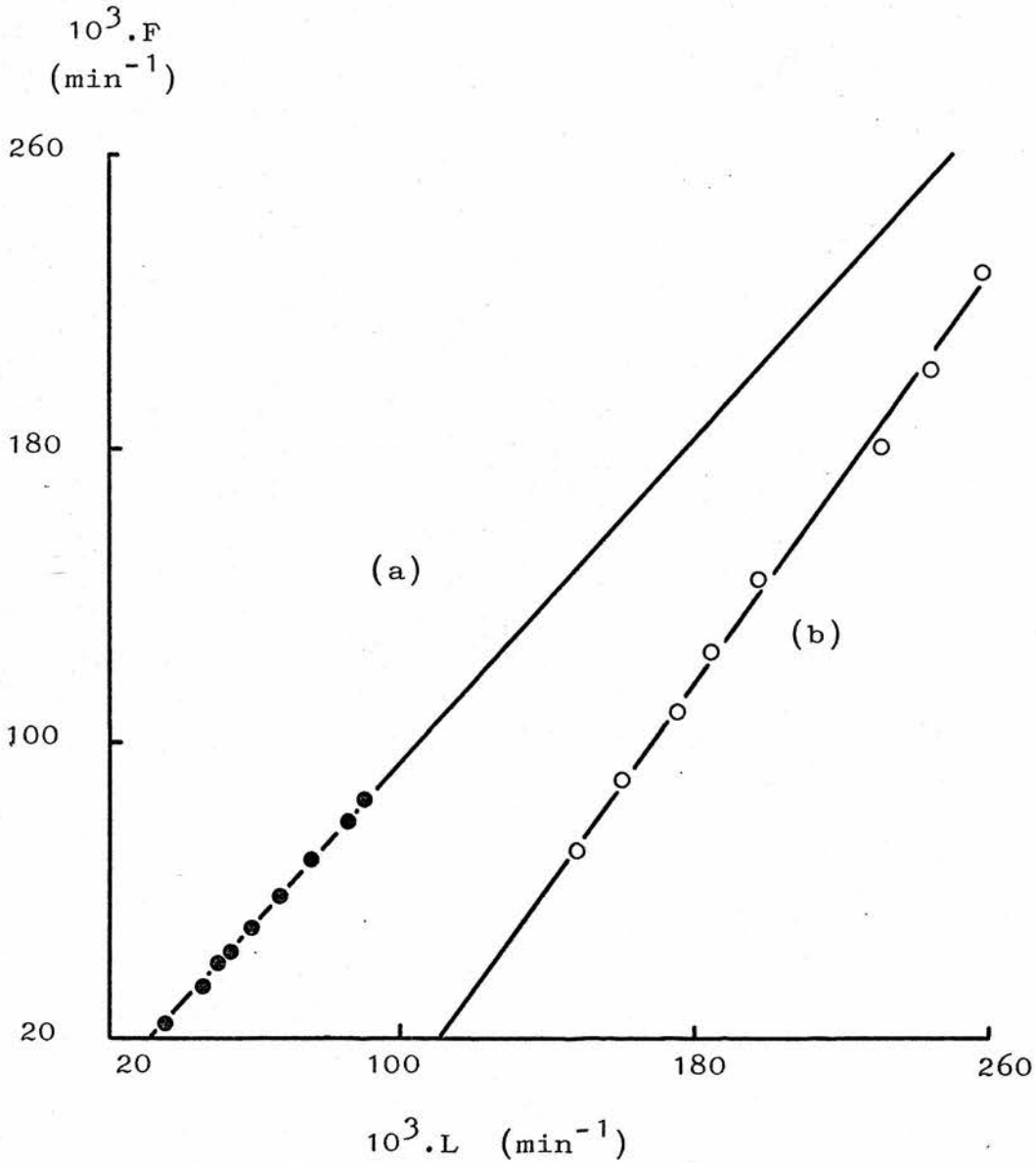
The Permeation of L(+) Arabinose: Typical Time-Courses Analysed

Mean concn. $\pm$ S.E.M. ( $\bar{x}$ mM)	258.1 $\pm$ 0.2	250.0 $\pm$ 0.2	81.6 $\pm$ 0.1	76.1 $\pm$ 0.1
Temp. ( $^{\circ}$ C)	19.6	19.5	19.5	19.4
$b' \pm s(b')$	1.117 $\pm$ 0.009	1.130 $\pm$ 0.034	1.642 $\pm$ 0.052	1.396 $\pm$ 0.031
$10^3 \cdot a' \pm s(a') (\text{min}^{-1})$	-16.38 $\pm$ 0.66	-24.89 $\pm$ 3.36	-193.7 $\pm$ 11.0	-134.3 $\pm$ 6.6
$b'' \pm s(b'')$	0.895 $\pm$ 0.008	0.882 $\pm$ 0.027	0.606 $\pm$ 0.019	0.714 $\pm$ 0.016
$10^3 \cdot a'' \pm s(a'') (\text{min}^{-1})$	14.69 $\pm$ 0.48	22.30 $\pm$ 2.33	118.4 $\pm$ 3.1	96.6 $\pm$ 2.6
$c(f') (\%)$	1.0	2.5	3.2	2.3
$\frac{m+x}{x}$	2.038	2.072	4.284	4.522
$K' \pm s(K) (\text{mM})$	66.8 $\pm$ 5.4	71.3 $\pm$ 18.8	84.9 $\pm$ 7.0	43.6 $\pm$ 3.4
$V' \pm s(V) (\text{mM}/\text{min})$	45.5 $\pm$ 4.2	61.7 $\pm$ 18.7	50.2 $\pm$ 5.5	40.6 $\pm$ 4.0
$K'' (\text{mM})$	67.2	73.9	86.3	44.2
$V'' (\text{mM}/\text{min})$	45.4	61.2	50.5	40.6
$K (\text{mM})$	67.0	72.6	85.6	43.9
$V (\text{mM}/\text{min})$	45.05	61.5	50.4	40.6

The means, and the standard errors of the means, of K and V are:  
 $K = 67.3 \pm 8.7 \text{ mM}$ ;  $V = 49.5 \pm 4.8 \text{ mM}/\text{min}$ .  
 Mean temperature =  $19.5^{\circ}\text{C}$ .

Figure 4.01

L(+) Arabinose: Plots of F against L



Time-courses (a) and (b) are summarised in Table 4.04.

The concentrations of arabinose were:

(a) 258 mM ; (b) 76.1 mM.

The lines represent the first-order regressions of F on L.

Table 4.04

The Permeation of L(+) Arabinose: Typical Time-Courses Summarised

Mean concn.		258.1 $\pm$ 0.2	76.1 $\pm$ 0.1
$\pm$ S.E.M. ( $\bar{x}$ mM)			
Temp. ( $^{\circ}$ C)		19.6	19.4
No. of observations		9	8
Time (t min)	min.	1.25	1.0
	max.	25.00	11.0
f'	min.	0.106	0.227
	max.	0.595	0.807
Mean recovery	arabinose	98.7 $\pm$ 0.3	99.2 $\pm$ 0.5
$\pm$ S.E.M. (%)	inulin	99.5 $\pm$ 0.2	100.1 $\pm$ 0.3

Each value of f' was the mean of two estimates.

#### 4.02 L(+) Arabinose

Time-courses were determined at virtually the same temperature for four different concentrations of extracellular arabinose. Two examples are given in Table 4.04. High concentrations of arabinose had to be used, to ensure that the rate of net permeation was sufficiently slow for accurate measurement. This precluded an analysis of the time-courses in terms of the Bulk Diffusion Hypothesis. Analysis in terms of the other two hypotheses is presented in Figure 4.01 and Table 4.05.

The plots of  $F$  against  $L$  seem to be linear. The mean coefficients of variation of  $f'$ , calculated from the regressions of  $F$  on  $L$ , are about two or three times those calculated from the known variability of the methods used to estimate pentoses and inulin (See Table 2.03, facing p. 64). Since each value of  $b'$  lies between  $m+x/x$  and unity, the kinetics are consistent with those of facilitated diffusion and not those of simple diffusion. Further evidence for the consistence is afforded by the four sets of values of  $K$  and  $V$ . They are of comparable magnitudes, although they were derived from time-courses determined at concentrations of extracellular arabinose which differed by some 250%.

Therefore it may be concluded that the factor governing the rate of permeation of  $L(+)$  arabinose at  $19.5^{\circ}\text{C}$  could be a "carrier" located in the plasma membrane. The means, and the standard errors of the means, of the characteristic parameters are:

$$K = 67.3 \pm 8.7 \text{ mM}; \quad V = 49.5 \pm 4.8 \text{ mM/min.}$$



Table 4.08

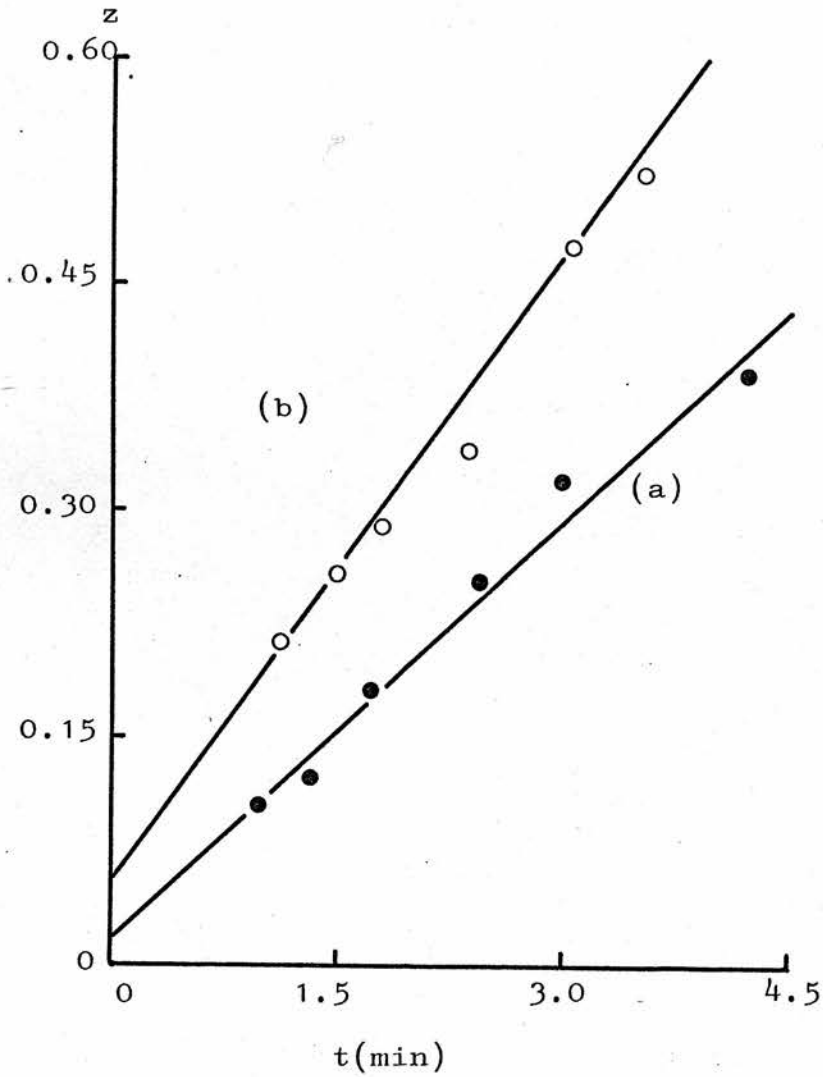
The Permeation of D(+) Glucose: Typical Time-CoursesAnalysed

Time-course	(a)	(b)
$b' \pm s(b')$	$1.165 \pm 0.089$	$1.148 \pm 0.055$
$10^3 \cdot a' \pm s(a') (\text{min}^{-1})$	$-139 \pm 29$	$-252 \pm 27$
$b'' \pm s(b'')$	$0.839 \pm 0.064$	$0.863 \pm 0.041$
$10^3 \cdot a'' \pm s(a'') (\text{min}^{-1})$	$124 \pm 16$	$222 \pm 13$
$c(f') (\%)$	4.6	2.8
$\frac{m + x}{x}$	6.78	12.6
$K' \pm s(K) (\text{mM})$	$9.3 \pm 5.0$	$3.8 \pm 1.4$
$V' \pm s(V) (\text{mM}/\text{min})$	$47.0 \pm 27.4$	$45.7 \pm 17.7$
$K'' (\text{mM})$	10.8	4.0
$V'' (\text{mM}/\text{min})$	44.2	44.0
$K (\text{mM})$	10.0	3.9
$V (\text{mM}/\text{min})$	45.6	44.8
$10^3 \cdot p \pm s(p)$	$91.5 \pm 7.1$	$134 \pm 5$
$10^3 \cdot q \pm s(q)$	$21.2 \pm 17.9$	$58.9 \pm 12.1$
$c(f') (\%)$	8.5	4.9
$F_{1,4}$	1.7	—
$10^3 \cdot D/e^2 \pm \text{S.E.} (\text{min}^{-1})$	$\frac{99.1}{158} \pm \frac{3.6}{5}$	—

$c(f')$  is the coefficient of variation of  $f'$ , and  $F_{1,4}$  is the variance ratio used to establish whether the addition of a term in  $t^2$  improved the fit of the first-order regression of  $z$  on  $t$ .

Figure 4.03

D(+) Glucose: Plots of  $z$  against  $t$



Time-courses (a) and (b) are summarised in Table 4.07.

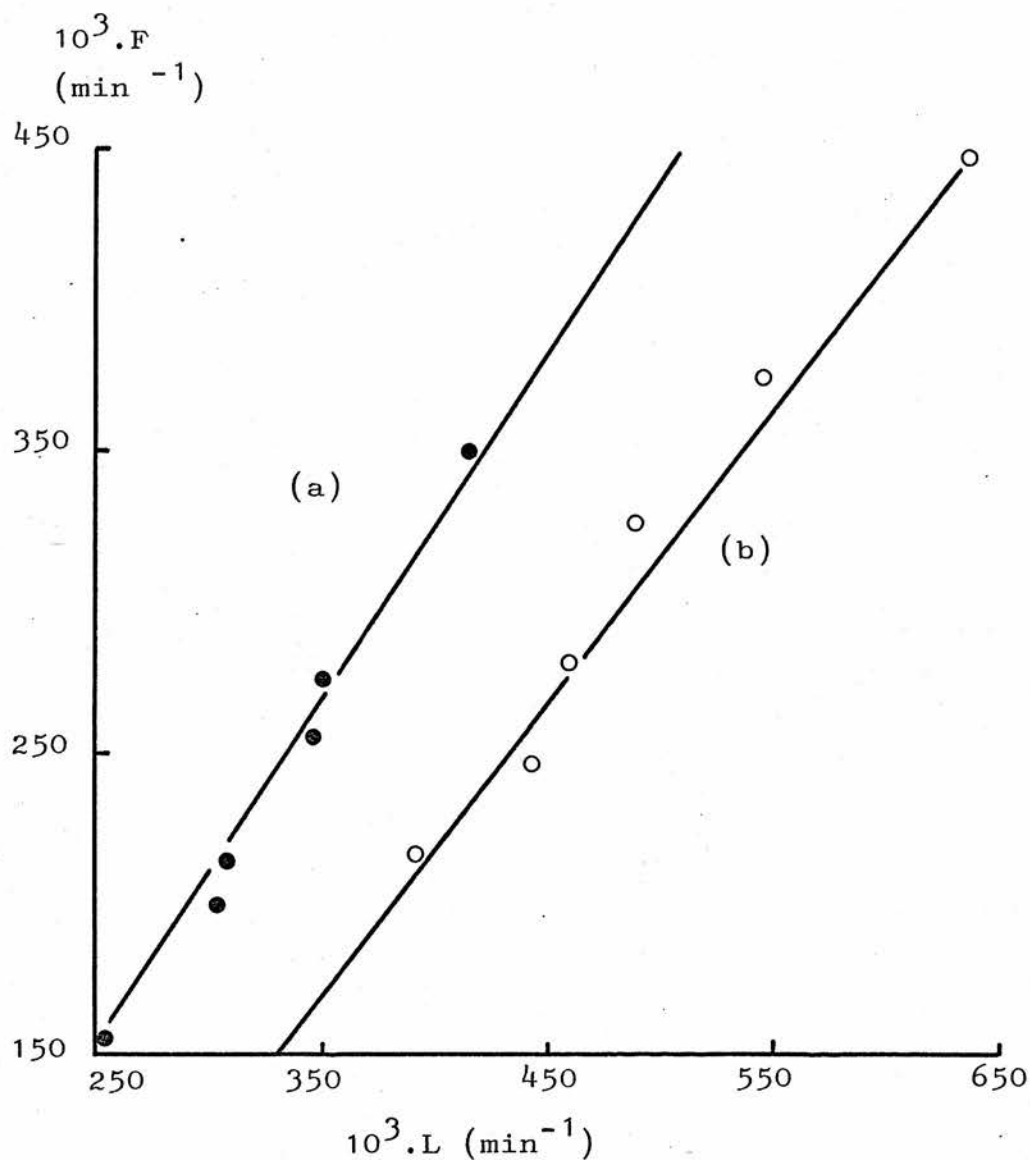
The concentrations of glucose were:

(a) 46.4 mM ; (b) 23.1 mM.

The lines represent the first-order regressions of  $z$  on  $t$ .

Figure 4.02

D(+) Glucose: Plots of F against L



Time-courses (a) and (b) are summarised in Table 4.07.

The concentrations of glucose were:

(a) 46.4 mM ; (b) 23.1 mM .

The lines represent the first-order regressions of F on L.

Table 4.07

The Permeation of D(+) Glucose: Typical Time-Courses Summarised

Time-Course	(a)	(b)
Mean Conc'n.	46.4 $\pm$ 0.3	23.1 $\pm$ 0.1
$\pm$ S.E.M. ( $\bar{x}$ mM)		
Temp. ( $^{\circ}$ C)	23.9	24.3
No. of observations	6	6
Time (t min) min.	0.95	1.10
max.	4.22	3.53
f' min.	0.332	0.490
max.	0.654	0.747
Mean recovery glucose	100.3 $\pm$ 0.4	100.8 $\pm$ 0.6
$\pm$ S.E.M. (%) inulin	101.0 $\pm$ 0.2	101.5 $\pm$ 0.4

Each value of f' was the mean of two estimates.

Table 4.06

The Permeation of D(+) Xylose: the Parameters Characterising  
the Membrane Carrier

Temp. (°C)	Concn.of Xylose (mM)	K $\pm$ s(K) (mM)	V $\pm$ s(V) (mM/min)
19.4	161	30.4 $\pm$ 9.3	69.4 $\pm$ 23.3
19.1	106	19.5 $\pm$ 3.5	48.2 $\pm$ 9.3
19.3	79.9	21.8 $\pm$ 6.3	42.2 $\pm$ 12.8

The means, and the standard errors of the means, of K and V are:

$$K = 23.9 \pm 3.3 \text{ mM} ; \quad V = 53.3 \pm 8.3 \text{ mM /min.}$$

Mean temperature = 19.3°C.

#### 4.03 D(+)Xylose

Three time-courses were determined at temperatures between 19.1°C and 19.4°C, and at different concentrations of extracellular xylose. Their analysis is not presented in detail, because it gave results that are similar to those in Section 4.02. Table 4.06 shows that the three sets of values of K and V are finite and positive, and of the same order of magnitude. Hence the kinetics of permeation conform to those predicted from the Simple Membrane Carrier Hypothesis. The means, and the standard errors of the means, of K and V are:

$$K = 23.9 \pm 3.3 \text{ mM} ; \quad V = 53.3 \pm 8.3 \text{ mM/min.}$$

#### 4.04 D(+)Glucose

Three time-courses were determined, between 23.5°C and 24.3°C, at concentrations of extracellular D(+) glucose which varied from 23.1 mM to 46.4 mM. Two are summarised in Table 4.07, and analysed in Figures 4.02 and 4.03, and Table 4.08. Neither conforms to a time-course of Simple Diffusion. Time-course (a) is consistent with both the Membrane Carrier and the Bulk Diffusion Hypotheses. However, the permeation equation derived from the latter hypothesis cannot be fitted to time-course (b): in this instance, q is significantly different from zero ( $P < 0.05$ ). Therefore the kinetics of permeation are not those of Bulk Diffusion. Conversely, the time-courses give estimates of V which are of similar magnitudes. The estimates of K differ by



Table 4.09

The Permeation of D(+) Glucose: the Parameters  
Characterising the Membrane Carrier

Temp. (°C)	Concn. of glucose (mM)	K $\pm$ s(K) (mM)	V $\pm$ s(V) (mM/min)
23.9	46.4	10.0 $\pm$ 5.0	45.6 $\pm$ 27.4
23.5	36.1	5.2 $\pm$ 3.6	79.9 $\pm$ 83.0
24.3	23.1	3.9 $\pm$ 1.4	44.8 $\pm$ 17.7

The means, and the standard errors of the means, of K and V are:

$$K = 6.4 \pm 1.9 \text{ mM} ; V = 56.8 \pm 11.6 \text{ mM/min.}$$

Mean temperature = 23.9°C.

6.1 mM: but the apparent standard error of the difference is 5.2 mM. The conclusion that the time-courses are explicable in terms of the Membrane Carrier Hypothesis is strengthened by an inspection of the estimates in Table 4.09. Their means, and the standard errors of their means, are:

$$K = 6.4 \pm 1.9 \text{ mM} ; \quad V = 56.8 \pm 11.6 \text{ mM/min.}$$

Two factors have been ignored in the calculation of the means. The first is that since erythrocytes metabolise glucose, all the estimates of  $f'$  would tend to be low. However, neither of the percentage recoveries of glucose given in Table 4.07 differs from 100% (Student's t-test;  $P > 0.05$ ).

The second factor is that the time-courses were determined over a range of temperature. In consequence, they are not strictly comparable. However, the range is only  $0.8^{\circ}\text{C}$ , which cannot have had an appreciable effect (see Section 5).

#### 4.05 Discussion

Neither of the Diffusion Hypotheses can account for the rates at which the three aldoses were observed to permeate the human erythrocyte. The Simple Membrane Carrier Hypothesis may do so; but the apparent standard errors of  $K$  and  $V$  are too large for the proof to be rigorous.

The magnitude of these standard errors requires explanation. Since  $K$  depends on  $(b'-1)$ , it is the precision of  $(b'-1)$  rather than of  $b'$  itself that determines the precision of  $K$ . Inspection of Tables 4.05

and 4.08 shows that  $b'$  varied from 1.1 to 1.7 and that its standard error was never more than 0.09. Thus, although the coefficient of variation of  $b'$  was less than 9%, that of  $(b'-1)$  could be an order of magnitude larger. The size of  $(b'-1)$  could have been increased by determining the time-course at a lower concentration of extracellular aldose,  $x$ . But the time to reach a given fractional penetration would then have decreased. The concentrations finally selected were a compromise: they made the rates of net permeation measurable, and yet gave values of  $b'$  that were appreciably greater than unity. It will be recalled that the analysis of the simulated time-courses described in Chapter III of Part Two gave estimates of  $K$  and  $V$  which are as imprecise as those presented in this Section. Hence the variability of the latter estimates does not necessarily imply either that the data on which they are based are unreliable, or that the kinetics of permeation are not those of the Carrier Hypothesis. Instead, it may only reflect the use of high concentrations of extracellular aldose.

The difference in the rates of penetrations of a - and b - D(+) glucose presents a further problem, because it implies that "mutarotated" glucose cannot be considered a single compound. In consequence, the analysis of the time-courses of permeation given in the preceding subsection is incomplete. However, in Chapter III of Part Two (pp.48 - 49 ) it is shown that, if the two anomers competed for a common carrier and had different values

of K but the same value of V, and if they were always at mutarotational equilibrium when within the cell, the permeation equation, 2.32, would fit the time-course of permeation. Some justification for the former of these suppositions is presented in the next Section.

Table 4.10

The Permeation of the Aldoses: the Temperature - Dependence  
of K and V

Aldose	Temp. (°C)	K (mM)	V (mM/min)	No. of determinations
L(+) arabinose	7.0	58.1	2.7	2
	19.5	67.3	50	4
	25.5	77.8	114	2
D(+) xylose	8.2	30.0	2.4	2
	19.3	23.9	53	3
	25.5	25.2	101	2
D(+) glucose	2.0	9.6	1.1	1
	4.0	10.4	2.0	2
	8.2	7.9	2.8	1
	13.9	8.4	9.6	1
	18.7	6.2	33	2
	23.9	6.4	57	3

## Section 5 Time-Courses of Permeation at Different Temperatures

Time-courses of permeation were determined for each of the three aldoses at a series of temperatures between  $2.0^{\circ}\text{C}$  and  $25.5^{\circ}\text{C}$ . With two exceptions, they were consistent with the Simple Membrane Carrier Hypothesis. The estimates of  $K$  and  $V$  calculated from them are summarised in Table 4.10. The apparent standard errors of the parameters have not been tabulated, because they gave coefficients of variation that are roughly constant.

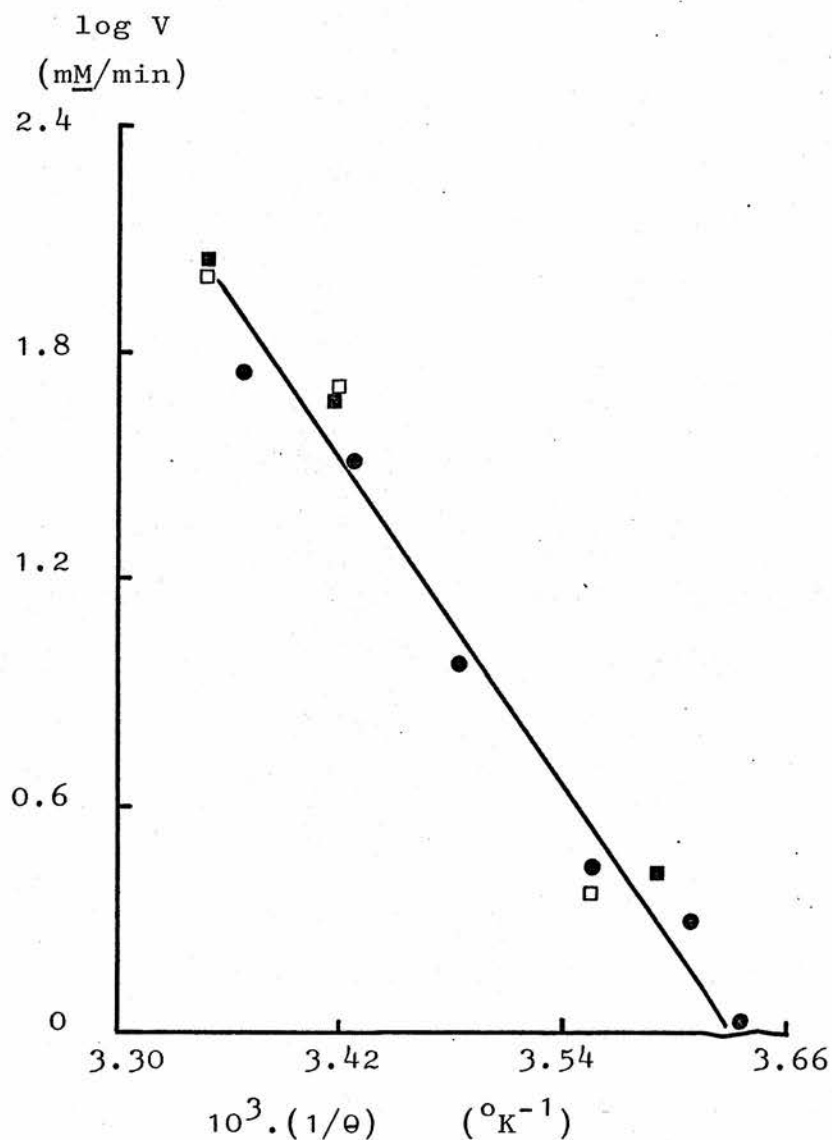
In the two exceptions, the concentration of extracellular arabinose was about  $350\text{ mM}$ , and the values of  $b'$  were less than unity. In one of the instances, microscopic examination showed that the cells were crenated by incubation with arabinose for 3 h at  $25.5^{\circ}\text{C}$ . It is possible that the exposure of the erythrocytes to a medium of such a high osmolality had made their haemoglobin crystallise. In the other instance, the theoretically unexpected value of  $b'$  was attributed to experimental error. Both exceptions were ignored.

Inspection of Table 4.10 shows that while all the values of  $V$  rose in like manner with temperature, there was no consistent variation in  $K$ . The  $K$  for arabinose increased by nearly  $20\text{ mM}$  when the temperature was changed from  $7.0^{\circ}\text{C}$  to  $25.5^{\circ}\text{C}$ . However, since the standard deviation of each estimate is some 10 to  $20\text{ mM}$  (see Table 4.05), the rise is not statistically significant. Similarly, the inverse relationship between temperature and the  $K$  for glucose is not significant.



Figure 4.04

The Aldoses: the Temperature - Dependence of V



$\theta$  is the absolute temperature ( $^{\circ}\text{K}$ ).

The aldoses are: D(+) glucose ●

L(+) arabinose ■

D(+) xylose □

The line through the points is defined by equation (4.01).

The temperature - dependence of V is illustrated by Figure 4.04, which is a plot of  $\log V$  against the reciprocal of the absolute temperature,  $\theta^{\circ}\text{K}$ . Since all the points seem to fall on the same straight line, it may be concluded that at any one temperature the aldoses have the same V. This conclusion supports the postulate that the aldoses share a common carrier for which  $k_3 < k_2$ . The unweighted first-order regression of  $\log V$  on  $1/\theta$  is:

$$\log V = 26.1 (\pm 1.3) - 7.2 (\pm 0.4) \cdot 10^3/\theta \quad (4.01)$$

The energy of activation for translocation across the membrane, calculated from the regression, is 33,000 kcal/mol.

It must be emphasised that V has been expressed as mmol aldose transferred /l intracellular water at isosmolality /min; or, for short, as mM/min. Since the volume of an erythrocyte which is suspended in a solution of fixed osmolality decreases with increasing temperature (Jacobs & Parpart, 1931), the values of V are not strictly comparable. However, because the percentage changes in volume are small when compared with the coefficient of variation of V, the individual values of V have not been corrected.

Section 6

The Effect of Insulin on the Permeation of  
Glucose

Although Park et al. (1956), and Rieser (1967), have stated that the permeability of the human erythrocyte to glucose is not affected by insulin, it is conceivable that any preparation of washed erythrocytes contains a physiologically significant amount of endogenous hormone. For this reason, an attempt was made to corroborate the above statement by determining the permeability of cells that had been pre-incubated with anti-insulin serum.

One millilitre of a 40% suspension of washed erythrocytes was preincubated for 1 h at 23.5°C with 1  $\mu$ l of anti-insulin serum. The serum was reconstituted "Insulin Neutralising/Precipitating Serum" (Burroughs Wellcome & Co., London), 1 ml of which is said to neutralise at least one international unit of ox insulin. After this pre-incubation, a time-course was determined in the usual way. The nature of the data was unexpected, in that  $f'$  did not always increase with time. Furthermore, about 15% of the cells had haemolysed after 30 min of incubation with glucose. Therefore the experiment was repeated with erythrocytes from a different donor. In this instance, some 10% of the erythrocytes had haemolysed by the end of the pre-incubation. In consequence, a time-course was not determined.

This preliminary investigation was not pursued further. Thus no attempt was made either to identify the component of the serum that had caused haemolysis, or to measure the permeability of erythrocytes to glucose in the presence of added insulin.

Section 7

Discussion

The results presented in this Chapter are consistent with the postulates:

- (i) that the aldoses cross the erythrocyte membrane in combination with some kind of "carrier";
- (ii) that they share a common carrier; and
- (iii) that the step which determines the overall rate of translocation is independent of the aldose being translocated, and is the transit of the carrier-aldose complex through the membrane.

However, because the carrier appears to distinguish between a - and b - D (+) glucose, the value of K that characterises the permeation of mutarotated glucose may be composite. If K(m), K(a) and K(b) refer to mutarotated glucose, a - glucose and b - glucose, respectively, and if mutarotated glucose contains 0.33 moles of a - glucose to 0.67 moles of b - glucose, it follows from equation (2.31) which is derived on pp. 48-49, that:

$$K(m) = \frac{K(\underline{b}) \cdot K(\underline{a})}{0.33 \cdot K(\underline{b}) + 0.67 \cdot K(\underline{a})}$$

Furthermore, the carrier may also differentiate between the anomers of L (+) arabinose and D (+) xylose. Therefore the parameters calculated for these compounds may also be composite.

If V is the same for all the anomers, and if the

intracellular anomers are always at mutarotational equilibrium, then the relative rates of penetration of a - and b - glucose are explained by assuming that  $K(\underline{a})$  is greater than  $K(\underline{b})$ . In this circumstance, the rate of influx of b - glucose is greater than that of a - glucose, at any one concentration of extracellular anomer. On the other hand, the rate of efflux is dependent only on the total concentration of intracellular glucose. Thus, for given extracellular and intracellular concentrations, the rate of net influx of b - glucose is greater than that of a - glucose.

Additional evidence for this difference between the values of  $K$  for a - and b - glucose is a correlation observed by LeFevre (1961a): that, for the monosaccharides,  $K$  increases as the stability of the C1 conformer decreases. It may be concluded from LeFevre's Figure 2 that the C1 conformer of b - D (+) glucose is more stable than that of the a - anomer. The same is true for b - and a - D (+) xylose, whereas the converse holds for b - and a - L (+) arabinose. Hence the  $K$  for a - D (+) xylose should be greater than that for b - D (+) xylose, whilst the opposite should be true for a - and b - L(+) arabinose.

The possibility that the hypothetical carrier differentiates between the anomers of the monosaccharides does not appear to have been entertained in most of the studies of the permeability of the human erythrocyte to this class of compounds. However, Feuchtwanger (1960) inferred that there was no difference between the rates of permeation, because erythrocytes did not disturb the equilibrium of extracellular a - and b - glucose. On

the other hand, Faust (1960) showed that the half-time of equilibration of b - glucose was less than that of a - glucose: which agrees with the finding of the present investigation.

This finding (as opposed to the conclusion drawn from it) does not depend on the validity of the Simple Membrane Carrier Hypothesis. However, two of the postulates made in the derivation of the permeation equation, 2.32, have been queried. The first is that  $k_3 < k_2$ ; the second is that the free carrier and the carrier-permeant complex have the same rate-constant for crossing the membrane (i.e. that  $k_3 = k_{+2}$ ,  $k_{-2} = k_{+4}$ ,  $k_{-4}$ ; see Figure 2.01, facing p. 46). They must be examined because, if they cannot be sustained, the values derived for K and V may be meaningless.

Evidence which favours the first postulate is the nature of the temperature - dependence of V. It will be recalled that the expressions for K and V, which are first given on p. 47, are:

$$K = \frac{k_2 + k_3}{k_1} \quad (2.26)$$

$$V = \frac{N}{2 \cdot w_o} \cdot \frac{k_2 \cdot k_3}{k_2 + k_3} \quad (2.27)$$

Hence, if  $k_3$  were not appreciably less than  $k_2$ , V would be determined by both  $k_2$  and  $k_3$ , and not by  $k_3$  alone. Since K is also dependent on  $k_2$  and  $k_3$ , it is unlikely that the aldoses would have different values of K but the same value of V. In addition, it is improbable that the



temperature - dependence of the common value of V would be the same. But it is evident that if  $k_2$  and  $k_3$  were of the same order of magnitude, values of K and V which were derived on the assumption that  $k_3$  is much less than  $k_2$  may not be valid. Hence before conclusions can be drawn from such values, their validity must be established.

If  $k_2$  and  $k_3$  were of the same order of magnitude, the permeation equation would have to be derived by integrating equation (2.28) and not equation (2.29) (see p. 47);

$$\text{i.e. from } \frac{di}{dt} = w_o \cdot KV \cdot \frac{(x - y)}{(K+x) \cdot (K+y) - (k_3/k_1)^2} \quad (2.28)$$

Integration of equation (2.28), and insertion of the boundary condition that  $i = 0$  when  $t = 0$  gives:

$$F = \frac{(m+x) \left( (K+x)^2 - (k_3/k_1)^2 \right)}{\left( (K+x)(K+m+x) - (k_3/k_1)^2 \right) \cdot x} \cdot L - \frac{m \cdot K \cdot V}{\left( (K+x)(K+m+x) - (k_3/k_1)^2 \right) \cdot x} \quad (4.02)$$

Hence the relationship between F and L would still be of the first order, but K and V could not be calculated from it. However, if the extreme circumstance, that  $k_3 \gg k_2$ , were true:

$$K = k_3/k_1$$

and, from equation (4.02):

$$K = (b'-1) \cdot \frac{x \cdot (m+x)}{2(m+x) - b' \cdot (m+2x)} \quad (4.03)$$

$$V = -a' \cdot \frac{(K(m+2x) + x(m+x)) \cdot x}{K \cdot m} \quad (4.04)$$

The usual equations for K and V, which are derived from equation (2.29), are (see p. 58):

$$K = (b'-1) \cdot \frac{x \cdot (m+x)}{m - (b'-1) \cdot x}$$

$$V = -a' \cdot \frac{(K+m+x) \cdot (K+x) \cdot x}{K \cdot m}$$

Thus, if K and V were obtained from the latter pair of equations, the values would be wrong.

The percentage errors in these values, when they are calculated from a glucose time-course, can be estimated as follows. Typically,  $K = 0.025.m$ ,  $x = 0.1.m$ , and  $b' = 1.15$ , so that the ratio of the true to the calculated value of K,  $R(K)$ , is:

$$R(K) = \frac{m - (b' - 1)x}{2(m+x) - b' \cdot (m+2x)} = 1.20$$

Similarly, the ratio of the true to the calculated value of V,  $R(V)$ , is:

$$R(V) = \frac{K(m+2x) + x(m+x)}{(K+m+x) \cdot (K+x)} = 1.04$$

It is evident that both K and V would be underestimated, but by only relatively small amounts. It is easy to show that the same conclusion holds for both arabinose and xylose. Hence it is arguable that whether or not  $k_3 < k_2$ , the values of K and V calculated on the assumption that the inequality holds are nearly equal to their true values. In consequence, the common value of V for the aldoses may be used as evidence that  $k_3 < k_2$ .

Nevertheless, the evidence is not conclusive, because the expressions for K and V are both symmetrical in  $k_2$  and  $k_3$ . Thus the common value of V merely indicates that  $k_2$  and  $k_3$  are of different orders of magnitude, and that the value of the smaller rate-constant is the same for all three aldoses. However,  $k_2$  is the rate-constant for the dissociation of the

carrier-aldose complex, and might be expected to depend on the aldose dissociating. Conversely,  $k_3$  is the rate-constant for the transfer of the carrier or the carrier-aldose complex across the membrane, and could well be independent of the aldose being transferred.

Sen & Widdas (1962), and Dawson & Widdas (1964), have described and discussed the temperature - dependence of the efflux of glucose from the human erythrocyte. Their values of  $V$  are quantitatively similar to those of this investigation. A plot of  $\log V$  against  $1/\theta$  was not linear, but had a slope which decreased with increasing  $1/\theta$ : see Figure 4 of Dawson & Widdas (1964). However, inspection of this Figure shows that the slope of the graph is almost constant in the temperature range  $18^\circ\text{C}$  to  $7^\circ\text{C}$ . Moreover, its magnitude is nearly the same as that of the plot in Figure 4.04 (facing p.158).

Dawson & Widdas explained the curvature of their plot by supposing that  $k_3$  is rate-limiting at  $37^\circ\text{C}$ , but that it becomes comparable in magnitude with  $k_2$  at lower temperatures. The explanation cannot be reconciled with the postulate, considered above, that  $k_3$  is always much less than  $k_2$ . An alternative explanation is that the physical properties of the erythrocyte membrane may be critically dependent upon temperature. For example, Chapman & Wallach (1968) have pointed out that a phospholipid has a clearly - defined transition temperature which is in the physiological range. The

temperature is determined by the chemical nature of the phospholipid and by the composition of its environment. This topic is referred to again in the General Discussion.

The second postulate to be examined is that the free carrier and the carrier-aldose complex have the same rate-constant for crossing the membrane. Stein and his colleagues (Levine, Oxender & Stein, 1965; Levine & Stein, 1966; Stein, 1967) have maintained that, in fact, the rate-constant for the complexed carrier, hereafter called  $k_3$ , is greater than that for the free carrier, hereafter called  $k_4$ ; and that both  $k_3$  and  $k_4$  are small compared with  $k_2$ . They have deduced that the ratio of  $k_3$  to  $k_4$ , which they call  $r$ , is about 3 or 4 at 25°C. Britton (1964) has predicted from the Carrier Hypothesis that if  $k_3 = r.k_4$  and  $k_3 \ll k_2$ , then  $K = k_2/k_1$ , and

$$\frac{di}{dt} = w_o.k_3.N . K. \frac{x - y}{2K^2 + (1+r).K(x+y) + 2rxy}$$

When  $x \gg K$  and  $y = 0$ , so that  $\frac{di}{dt} = V$  :

$$V = k_3.N/(1+r)$$

$$\text{and } \frac{di}{dt} = w_o.KV \frac{(1+r).(x-y)}{2K^2 + (1+r).K(x+y) + 2r.xy} \quad (4.05)$$

Integration of equation (4.05), with the usual boundary condition that  $i = 0$  when  $t = 0$ , gives:

$$F = \frac{2(m+x)(K^2 + (1+r).Kx + rx^2)}{(2K^2 + (1+r).K(m+2x) + 2rx(m+x)).x} . L - \frac{(1+r).m.K.V}{(2K^2 + (1+r).K(m+2x) + 2rx(m+x)).x}$$

Therefore  $F$  would still be linearly related to  $L$ .

However, if apparent values of K and V were calculated from the relationship of F to L in the usual way (that is, assuming that  $k_3 = k_4$ , or that  $r = 1$ ), when in fact r is not equal to unity, then the apparent values of K and V, K(app) and V(app), would vary with x. For example:

$$K(\text{app}) = \frac{K(2K + (1+r).x)}{(1+r).K + 2r.x}$$

But the variation would be small. For instance, if r were equal to 3, and if x were very much less than K, K(app) would equal  $K/2$ . Conversely, if x were very much greater than K, K(app) would equal  $2K/3$ . The values of K presented in Section 4 are not sufficiently precise for such a small variation to be detected. Therefore the present experiments (which were not, in fact, designed to evaluate r) throw no further light on its magnitude. For these two reasons, the determination of r, and the consequences of its being different from unity, are only touched upon at the end of this Discussion - especially as they have been elaborated by Stein (1967).

Nevertheless, it must be emphasised at this juncture that the values which different workers quote for "K" and "V" are only directly comparable if r equals unity. However, Levine & Stein (1966) have shown that the apparent value of K calculated by Sen & Widdas (1962) equals  $(1+r).K/2r$ , so long as the concentration of intracellular glucose used is much greater than K. This condition is fulfilled at 25°C, but may be vitiated

Table 4.11

The Permeation of the Aldoses: a Comparison of the  
Published Estimates of K and V

Aldose	Temp. (°C)	K (mM)	V (mM/min)	Ref.
D(+) glucose	25.0	2.24	118	Sen & Widdas (1962)
	25.0	3.5	165	Levine & Stein (1966)
	23.9	6.4	66	this study
	20.0	1.68	74	Sen & Widdas (1962)
	8.2	7.9	3.2	this study ,
	7.0	0.58	15	Sen & Widdas (1962)
	5.0	0.50	1.1	Stein (1967)
	4.0	10.4	2.3	this study
D(+) xylose	37.0	59	630	LeFevre (1962)
	25.5	25	117	this study
L(+)arabinose	37.0	230	690	LeFevre (1962)
	25.5	78	132	this study

The values of V are expressed in terms of the volume of intracellular water at an osmotic pressure of 310 millosmolal.



at about  $10^{\circ}\text{C}$ . Their own estimates of  $K(\text{app})$ , and those of LeFevre (1962), were made from time-courses of net influx. In consequence, Levine & Stein's and LeFevre's values of  $K(\text{app})$  are directly comparable to the present values, and those of Sen & Widdas may well be so. The same is assumed to apply to the values of  $V(\text{app})$ .

A second factor which precludes the direct comparison of different workers' results is that the "isotone" is often used as the unit of quantity, and that  $V$  is always given in terms of the "isotonic" water content of the cell. Yet the "isotone" is not a well-defined quantity. For example, two recent estimates of the milliosmolality of human plasma are 282 (Savitz, Sidel & Solomon, 1964), and 285 (Williams, Fordham, Hollander & Welt, 1959). The suspension medium used in this investigation had a milliosmolality of 268, whereas that used by Sen & Widdas (1962) was 342 milliosmolal. Stein (1967) has collated, in his Table 4.3, most of the values of  $K$  and  $V$  that have been determined in different laboratories and in different experimental conditions; but he has assumed that in every instance the "isotone" was 310 milliosmoles.

The published values of  $K$  and  $V$  that are comparable to those of the present investigation are collected in Table 4.11. The units of  $K$  are  $\text{mM}$ , whilst those of  $V$  are  $\frac{\text{mmol}}{\text{mM}}/1$  intracellular water/min, the volume of the intracellular water being measured at an osmotic pressure of 310 milliosmolal. It is apparent that for  $D(+)$  glucose, the present values of  $K$  tend to be greater than those already published, whereas the opposite is true

of V. And although the comparable values of the parameters that characterise the permeation of the pentoses were determined at different temperatures, it is unlikely that temperature alone could account for the discrepancies between them.

There are several possible explanations for the variation between these estimates of ostensibly the same parameters. One is that the different methods used to estimate the parameters could have involved different systematic errors. For example, in the novel technique described in Part Three, it is conceivable that the mass of cells (in the lower compartment of a waisted centrifuge tube) was in effect isolated from the bulk of the suspension medium (in the upper compartment) before the plunger was inserted. If this were so, each "time" would be over-estimated by not more than 25 s. The consequence of an over-estimate of 6 s, in a typical glucose time-course, was explored as follows. A "perfect" glucose time-course was constructed, with  $K = 5$ ,  $V = 50$ ,  $m = 300$  and  $x = 35$ . Each "perfect" value of  $t$  was increased by 6 s, to form the "experimental" time-course.  $K$  and  $V$  were estimated from the "experimental" time course in the usual way. The estimates were:

$$K = 7.60 \quad s(K) = 0.19 \quad V = 38.6 \quad s(V) = 1.0$$

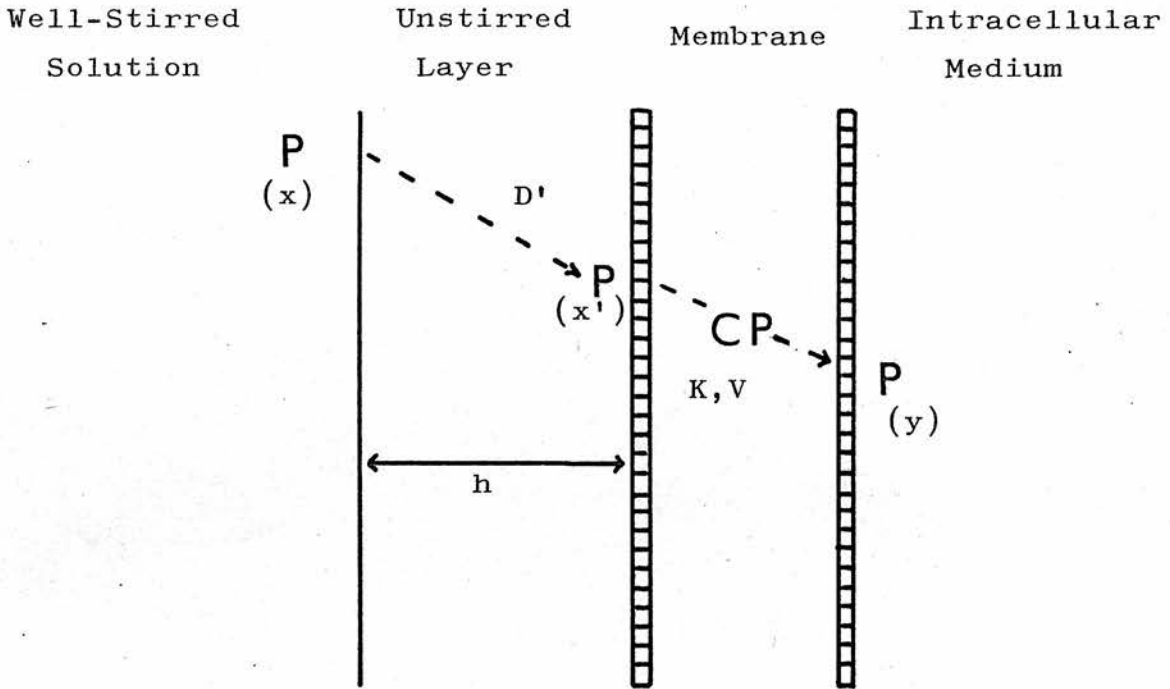
Hence, if each "time" were over-estimated, a plot of  $F$  against  $L$  would be nearly linear, but the estimate of  $K$  would be too great, whereas that of  $V$  would be too small. This systematic error could explain the signs

of the differences between the present and the published parameters for D(+) glucose, but not those for the pentoses. Furthermore, as temperature decreased, the period over which a time-course was determined increased. Therefore the magnitudes of the percentage differences should have decreased, and not increased.

A second admissible systematic error is the presence of an "unstirred layer" of medium at one or both of the surfaces of the membrane (Dainty, 1963). For example, Sha'afi et al. (1967) inferred that a layer of thickness  $5.5 \mu\text{m}$  surrounded erythrocytes in a turbulent stream; and Green & Otori (1970) showed that  $65 \mu\text{m}$  was the thickness of the layer separating the posterior surface of the rabbit cornea from the bulk of the well-stirred solution. However, if the erythrocyte were a disc of diameter  $8 \mu\text{m}$  and length  $2 \mu\text{m}$  (Whittam, 1964), surrounded by a layer of thickness  $6 \mu\text{m}$ , then the total volume of one cell plus its unstirred layer would be nearly 50 times that of the cell itself. In this study, the haematocrit of the cells being incubated with aldose was about 7%. Therefore each cell must have been less than one "unstirred layer" from its nearest neighbours. Presumably these neighbours would tend to "mix" the contents of the layer. On the other hand, since Sen & Widdas (1962) used cell suspensions of haematocrit 0.014%, their results could have been influenced by

Figure 4.05

A Membrane Surrounded by an Unstirred Layer



$P$  is the permeant, and  $C$  is a mobile carrier like that of Figure 2.01.

The osmolalities of the permeant in the well-stirred solution, at the outer surface of the membrane, and at the inner surface of the membrane are  $x$ ,  $x'$  and  $y$ , respectively.

$D'$  is the diffusion coefficient of the permeant in the unstirred layer of thickness  $h$ , and the carrier parameters are  $K$  and  $V$ .

the presence of unstirred layers in the extracellular medium. Therefollows an attempt to establish the effect of such layers.

Figure 4.05 depicts a plasma membrane, whose outer surface is surrounded by an unstirred layer of thickness  $h$ . The concentrations of permeant in the well-stirred extracellular compartment, at the outer surface of the membrane, and at the inner surface of the membrane, are  $x$ ,  $x'$  and  $y$ , respectively. The permeant has a diffusion coefficient of  $D'$ , and is transferred across the membrane by a system of facilitated diffusion of parameters  $K$  and  $V$ . At the steady state, the rate of net flux of permeant from the well-stirred extracellular compartment to the intracellular compartment,  $di/dt$ , is:

$$\frac{di}{dt} = \frac{D' \cdot A \cdot (x - x')}{h} = \frac{w_o \cdot K \cdot V \cdot (x' - y)}{(K + x') \cdot (K + y)}$$

where  $A$  is the surface area, and  $w_o$  is the isosmolal water content, of the cell.

It can be shown that:

$$\frac{(K+y)}{(P' \cdot A)} \cdot \frac{(di)^2}{(dt)} - \left( (K+x) \cdot (K+y) + \frac{w_o \cdot K \cdot V}{P' \cdot A} \right) \cdot \frac{(di)}{(dt)} + w_o \cdot K \cdot V \cdot (x - y) = 0$$

where  $P'$  is the permeability coefficient of the unstirred layer. Moreover, if  $w_o \cdot V/K$  is small compared with  $P' \cdot A$  (that is, if the permeant diffuses much more quickly through the unstirred layer than through the membrane):

$$\frac{di}{dt} = \frac{w_o \cdot K \cdot V \cdot (x - y)}{(K + x) \cdot (K + y) + (w_o \cdot K \cdot V / P' \cdot A)}$$

If the human erythrocyte has an isosmolal volume of

87  $\mu\text{m}^3$ , a surface area of 155  $\mu\text{m}^2$ , and is covered by an unstirred layer of 6  $\mu\text{m}$ ; and if the permeant is glucose, for which, at 18°C,  $K = 5 \text{ mM}$ ,  $V = 50 \text{ mM/min}$ , and  $D^i = 6 \times 10^{-6} \text{ cm}^2/\text{s}$  (Glasstone, Laidler & Eyring, 1941), then

$$\begin{aligned} \frac{h \cdot w_o \cdot K \cdot V}{D^i \cdot A} &= \frac{w_o \cdot K \cdot V}{P^i \cdot A} \\ &= \frac{6 \times 10^{-4} \times 87 \times 10^{-12} \times 5 \times 10^{-3} \times 50 \times 10^{-3}}{6 \times 10^{-6} \times 155 \times 10^{-8}} \\ &= 1.4 \text{ (mM)}^2 \end{aligned}$$

In the experiments of Sen & Widdas,  $y$  was initially 76 mM, so that  $(K+x) \cdot (K+y)$  was at least 400 (mM)<sup>2</sup>: that is, much greater than  $w_o \cdot K \cdot V / P^i \cdot A$ . It may be concluded that the presence of extracellular unstirred layers would not affect their estimates of  $K$  and  $V$  by very much - especially as the efflux of water from the cells would tend to stir the layers.

It is impossible to estimate the effect of an unstirred layer within the cell, because the diffusion coefficient of intracellular aldose is unknown. However, Perutz (1948) has concluded that at isosmolality an individual molecule of haemoglobin is just able to rotate freely. In hyperosmolal conditions, its rotation is restricted. Hence there may be only a limited number of aqueous channels by which a permeant can leave the inner surface of the membrane. Thus, whether or not the diffusion coefficient of permeant in the intracellular water is equal to that in aqueous solution, the effective rate of diffusion throughout the intracellular



volume may be reduced. If it is reduced,  $V$  is more likely to be underestimated when the erythrocytes are swelling to their isosmolal volume than when they are shrinking to this volume. In the present study, and in the studies of LeFevre (1962) and Levine & Stein (1966), swelling erythrocytes were used; conversely, Sen & Widdas (1962) observed cells that were shrinking. The last-named derived the greatest values of  $V$  for glucose at low temperatures.

Another possible explanation for the variation between different groups' estimates of ostensibly the same parameters is that they were not in fact studying the same type of cell. The composition of the membrane of the washed erythrocyte presumably depends both on the number of times that the cell was washed and on the composition of the medium in which it was washed. Yet each group has its own washing procedure and medium. The permeability of the erythrocytes may depend on the length of time for which they have been stored (Bican & Lacko, 1966a). Yet different groups study cells that have been stored in different conditions for different lengths of time. Laris (1967) has suggested that the permeability of the bovine erythrocyte to fructose is genetically determined. The same could be true of the permeability of the human erythrocyte to the aldoses. Finally, an individual donor's cells do not form a homogeneous population. Rather, many of the properties of each cell are dependent on its age (Pennell, 1964; Bican & Lacko, 1966b).

Therefore it may only be fair to compare estimates which were derived using different techniques when variables of the sort considered here have been either eliminated or allowed for.

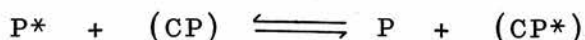
In summary, it is evident that although there are several possible experimental reasons for the differences between the estimates of K and V in Table 4.11, these differences remain unexplained. They may, therefore, point to an inadequacy in the Simple Membrane Carrier Hypothesis. For this reason, the Discussion is concluded with a short critique of the evidence for the postulate that the free and complexed carriers have different rate-constants for crossing the membrane (that is, that  $r$  does not equal unity). The evidence has been reviewed by Stein (1967).

The value of  $r$  can be calculated by comparing the maximum rates of unidirectional flux of labelled permeant measured in two extreme conditions. The conditions are, firstly, when the medium at the trans surface has a concentration of unlabelled permeant that saturates the carrier; and, secondly, when it contains no permeant. The ratio of the first rate of flux to the second rate of flux is  $(1+r)/2$ .

The calculation depends upon the exclusion of at least three possibilities. The first is a system for "exchange diffusion" - that is, a system which facilitates the obligatory exchange of a molecule of intracellular permeant for one of extracellular permeant.

Whittam (1964), and Lubowitz and Whittam (1969), have described such a system for  $\text{Na}^+$ . It is difficult to prove that there is no similar system for the monosaccharides.

The second possibility, which was suggested by Britton (1964), is an exchange reaction of the type.



where  $\text{P}^*$  represents the labelled, and  $\text{P}$  the unlabelled, permeant. This reaction would increase the rate of unidirectional flux of  $\text{P}^*$  if the rate constant for the dissociation of the complex,  $k_2$ , were small compared with that for its crossing the membrane,  $k_3$ . In this circumstance, the complex could cross the membrane several times before dissociating. However, the inference made above, that  $k_2$  is greater than  $k_3$ , argues against the existence of the possibility.

Miller (1968) has described the third possibility. He supposes that when  $\text{P}^*$  has reached the trans surface of the membrane and been released from the carrier, it does not diffuse rapidly away. Hence its concentration at the trans surface is greater than that in the bulk of the solution. In consequence, the rate of unidirectional flux of permeant from the trans to the cis surface is greater than would be predicted from the concentration of trans permeant. However, when unlabelled permeant,  $\text{P}$ , is also present at the trans surface, it competes with  $\text{P}^*$  for transfer back to the cis surface. The apparent rate of unidirectional flux of  $\text{P}^*$  from the cis to the trans surface is thereby increased.

The explanation is unsatisfactory for two reasons. Firstly, when P is absent from the trans medium, there is a net flow across the membrane of water as well as of P\*. This would tend to stir the contents of the "slow diffusion" layer at the trans surface. Conversely, if P were present at the trans surface, there would be no osmotic flow of water. Therefore the presence of P might decrease the flux of P\*, instead of increasing it.

Secondly, there is nothing to suggest that free P would diffuse rapidly away from the cis surface after it had crossed the membrane. Rather, it would tend to compete with P\* for transfer back to the trans surface, thus reducing the rate of flux of P\*. In this way P, initially present at the trans surface, might reduce still further the rate of flux of P\*.

Although these three possibilities have not been completely excluded, there is a considerable body of experimental evidence which favours the view that  $r$  does not equal unity. However, no one model can as yet completely account for all the evidence (Miller, 1968; Levine & Levine, 1969). The explanation for this inability may be that the models ignore the heterogeneity of erythrocytes, and the distinction the cells make between the anomers of the aldoses.

Chapter XVI THE PERMEATION OF MESO-ERYTHRITOL

Section 1 The Distribution at Equilibrium

The equilibrium distribution of erythritol between intra- and extra- erythrocyte water was determined in a manner similar to that for arabinose and xylose (see Chapter XV, Section 1). In this instance, 5 ml of a suspension of washed erythrocytes, of haematocrit 40%, were incubated for 6 h at 23°C with 2 ml of incubation medium containing a total of either (a) 200  $\mu\text{mol}$ , or (b) 20  $\mu\text{mol}$  of erythritol. The concentration of erythritol in the intracellular medium at the end of this period was expressed as a percentage of that in the extracellular medium. The means of these concentrations, and their standard errors, were:

$$(a) \quad 99.0 \pm 1.2$$

$$(b) \quad 99.5 \pm 0.8$$

Each is the mean of four determinations. Since neither differs significantly from 100% (Student's t-test;  $P > 0.05$ ), it was concluded that at equilibrium erythritol is uniformly distributed through the water of the suspension.

Table 4.13

The Permeation of Erythritol: the Time-Courses Analysed

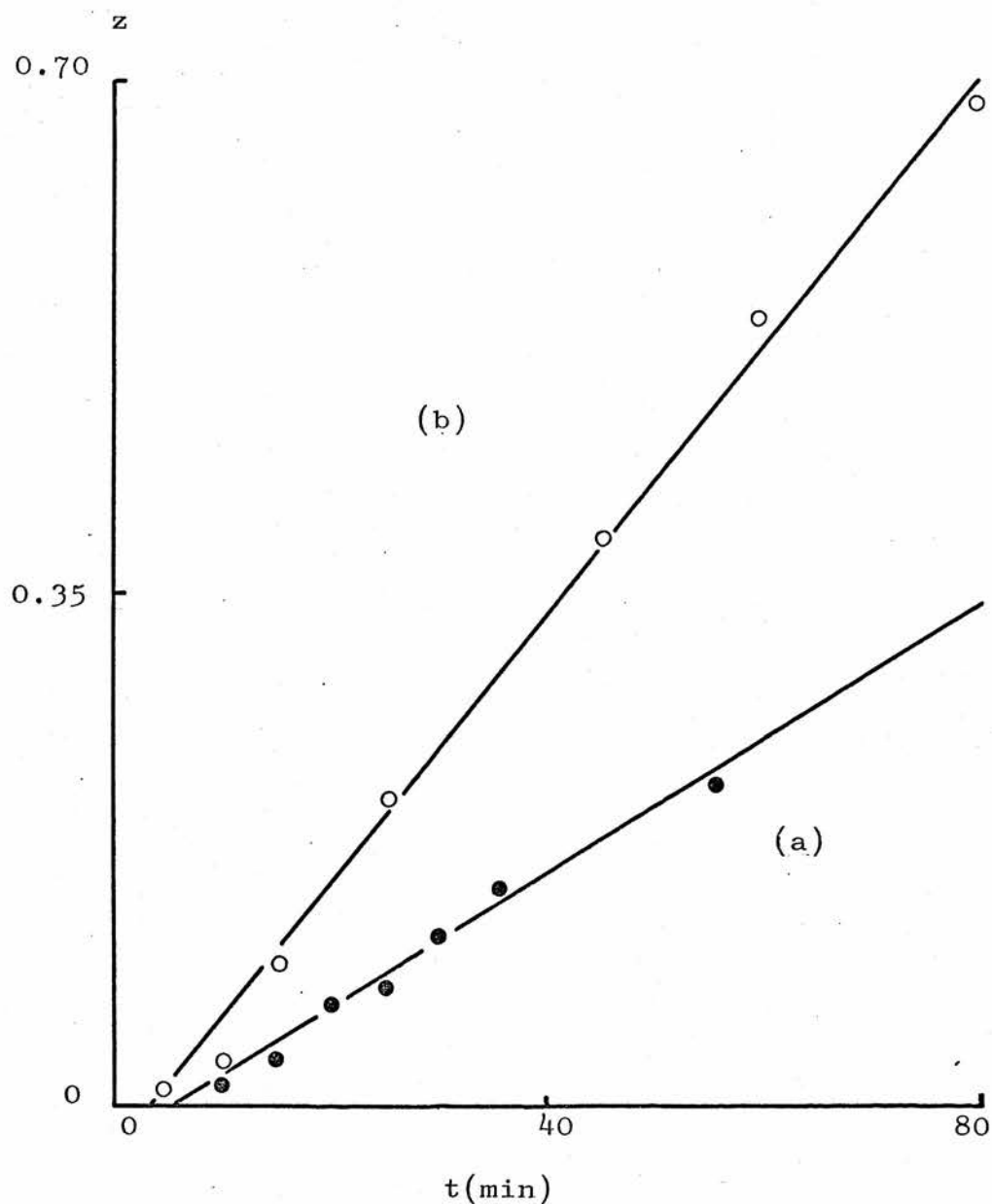
Time-course	(a)	(b)
$b' \pm s(b')$	$1.148 \pm 0.259$	$2.528 \pm 0.278$
$10^3 \cdot a' \pm s(a') \text{ (min}^{-1}\text{)}$	$-5.04 \pm 4.03$	$-50.6 \pm 7.8$
$b'' \pm s(b'')$	$0.696 \pm 0.156$	$0.462 \pm 0.085$
$10^3 \cdot a'' \pm s(a'') \text{ (min}^{-1}\text{)}$	$6.64 \pm 2.02$	$18.2 \pm 1.8$
$c(f') \text{ (\%)}$	7.8	8.6
$\frac{m + x}{x}$	7.20	62.7
$K' \pm s(K) \text{ (mM)}$	$7.7 \pm 13.4$	$6.9 \pm 1.2$
$V' \pm s(V) \text{ (mM/min)}$	$1.7 \pm 3.4$	$0.4 \pm 0.1$
$K'' \text{ (mM)}$	23.8	5.2
$V'' \text{ (mM/min)}$	1.5	0.3
$K \text{ (mM)}$	15.8	6.1
$V \text{ (mM/min)}$	1.6	0.4
$10^3 \cdot p \pm s(p)$	$4.55 \pm 0.24$	$9.19 \pm 0.21$
$10^3 \cdot q \pm s(q)$	$-21.5 \pm 7.4$	$-29.5 \pm 8.9$

$c(f')$  is the coefficient of variation of  $f'$ .



Figure 4.07

meso-Erythritol: Plots of  $z$  against  $t$



Time-courses (a) and (b) are summarised in Table 4.12.

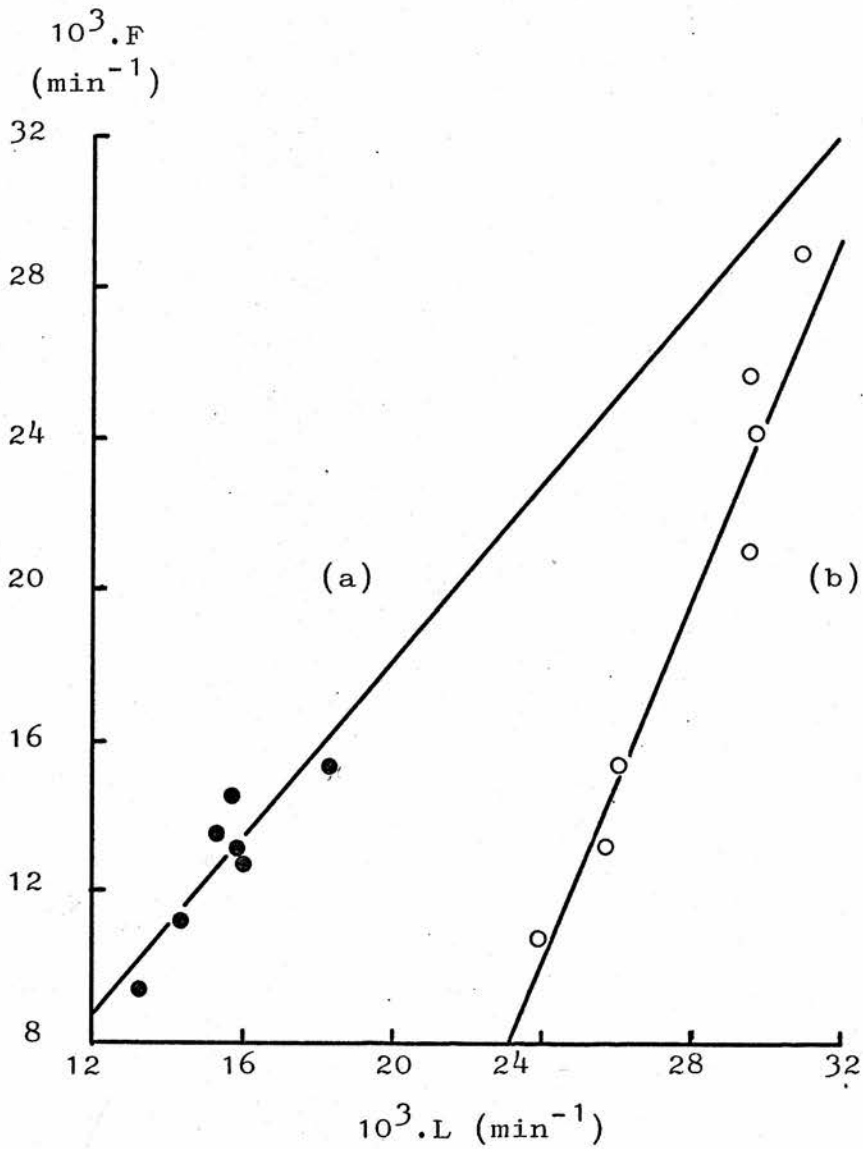
The concentrations of erythritol were:

(a) 43.5 mM ; (b) 4.35 mM.

The lines represent the first-order regressions of  $z$  on  $t$ .

Figure 4.06

meso-Erythritol: Plots of F against L



Time-courses (a) and (b) are summarised in Table 4.12.

The concentrations of erythritol were:

(a) 43.5 mM ; (b) 4.35 mM.

The lines represent the first-order regressions of F on L.

Table 4.12

The Permeation of Erythritol: the Time-Courses  
Summarised

Time-course	(a)	(b)
Temp. ( $^{\circ}\text{C}$ )	21.3	21.5
No. of observations	7	7
Mean Conc.		
$\pm$ S.E.M. ( $\bar{x}$ mM)	$43.5 \pm 0.2$	$4.35 \pm 0.01$
min.	10.0	4.5
Time (t min)		
max.	55.0	79.5
f'		
min.	0.145	0.130
max.	0.516	0.850
Mean recovery erythritol	$99.2 \pm 0.4$	$99.6 \pm 0.4$
$\pm$ S.E.M. (%) inulin	$101.1 \pm 0.3$	$99.5 \pm 0.1$

## Section 2      The Time-Course of Permeation

### 2.01    The Precision of an Estimate of $f'$

Six estimates of one observation ( $t$ ,  $f'$ ) were made for erythrocytes incubated at  $21.3^{\circ}\text{C}$  with a medium containing erythritol of final concentration  $43.5 \text{ mM}$ . The mean and its standard error were:

$$t = 30 \text{ min} \qquad f' = 0.381 \pm 0.014$$

Therefore the coefficient of variation of an individual value of  $f'$  was taken to be about 9%.

### 2.02    Time-Course at Different Concentrations of Extracellular Erythritol

Two time-courses of permeation were determined at virtually the same temperature but at concentrations of extracellular erythritol that differed by an order of magnitude. They are summarised in Table 4.12.

The results of the analyses of the two time-courses in terms of the three hypotheses are presented in Figures 4.06 and 4.07, and Table 4.13. Since, for both time-courses,  $b'$  is very different from  $m+x/x$ , and  $q$  is significantly different from zero ( $P < 0.05$ ), the observed kinetics of permeation are not those of either Simple Diffusion or Bulk Diffusion. On the other hand, they are consistent with the kinetics of a carrier-mediated process, which is characterised by a  $K$  of  $11.0 \text{ mM}$  and a  $V$  of  $1.0 \text{ mM/min}$ . But because the coefficients of variation of  $K$  and  $V$  are so large, the consistence can only be regarded as tentative.

There are two further items that merit comment. Firstly,  $p$  and  $q$  change with  $x$  in the manner predicted from the Membrane Carrier Hypothesis (see Part Two, Chapter V). Secondly, although the coefficients of variation of  $f'$  estimated from the regressions of  $F$  on  $L$  are nearly equal, the coefficients of variation of the two sets of characteristic parameters differ markedly. This is because the precision of a set of parameters is largely determined by the precision of  $(b'-1)$ , and hence the precision of the former increases with the magnitude of the latter.

## Section 3

Discussion

The lack of precision of the data precluded their accurate analysis. Hence assertions about the mechanism by which meso-erythritol permeates the human erythrocyte will only be made with confidence after the reliability of the method for estimating this compound has been enhanced.

Nevertheless, it seems that permeation is carrier-mediated. This agrees with a conclusion of Bowyer & Widdas (1955): that, because glucose inhibits the influx of meso-erythritol, the two compounds penetrate via the same system. However, the agreement is only superficial. If the aldoses and meso-erythritol shared a common carrier whose properties are those given in Chapter III of Part Two, all the compounds would have the same maximum rate of permeation,  $V$ . They do not. At  $21.5^{\circ}\text{C}$ , the aldoses have a  $V$  of about  $46 \text{ mM/min}$  (calculated from equation (4.01), p. 158) whereas the  $V$  for erythritol is about  $1.0 \text{ mM/min}$ .

This argument would be vitiated if the assumptions that were made about the relative magnitudes of the rate-constants for the four stages comprising carrier mediated transfer do not hold. In the preceding Chapter, it is concluded that, for the aldoses,  $k_3 < k_2$ , so that  $V$  is directly proportional to  $k_3$ . If erythritol and the aldoses had the same  $k_3$ , but the former had a  $k_2$  that was much less than the common  $k_3$ , its  $V$  would be directly proportional to  $k_2$ . However, it is also shown in the preceding Chapter that when  $k_3 \gg k_2$ ,  $K$  and  $V$  are given



by equations (4.03) and (4.04) respectively.

$$\text{i.e. } K = (b' - 1) \cdot \frac{x \cdot (m+x)}{2(m+x) - b' \cdot (m+2x)} \quad (4.03)$$

Since K must be positive:

$$b' > \frac{2(m+x)}{m + 2x}$$

In time-course (b),  $b' = 2.53$ , and  $\frac{2(m+x)}{m + 2x} = 1.97$

The hypothesis that  $k_3 > k_2$  is untenable.

Thus it is evident that the way by which meso-erythritol enters the human erythrocyte is still undefined. Persuasive evidence for carrier-mediated transfer would be a demonstration of the counter-transport of  $^{12}\text{C}$ -erythritol by  $^{14}\text{C}$ -erythritol. Studies of the permeation of d-, l- and meso-erythritol would give information about the stereo-specificity of transfer, and about the rate-constants describing its four stages. And the counter-transport of meso-erythritol by glucose would accord well with the contention that the two compounds share a common carrier.

Table 4.14

The Distribution of Thiourea at Equilibrium

Mean Concn. ± S.E.M.	Time (t min)	Mean f' ± S.E.M.	Mean Recovery ± S.E.M. (%)	No. of observations
( $\bar{x}$ mM)			thiourea      inulin	
400.6 ± 4.1	60	1.47 ± 0.01	100.0 ± 0.4    98.9 ± 0.3	3
244.3 ± 9.9	30	1.33 ± 0.03	99.4 ± 0.1    99.1 ± 0.2	8
110.7 ± 0.3	20	1.55 ± 0.01	101.5 ± 0.2    99.6 ± 0.2	6
18.4 ± 0.2	60	1.51 ± 0.02	98.6 ± 0.6    99.2 ± 0.2	3
8.9 ± 0.1	60	1.47 ± 0.03	99.5 ± 0.5    99.1 ± 0.3	4

Temperature = 23.5 - 24.0°C.

## Chapter XVII THE PERMEATION OF THIOUREA

The equilibrium distribution of thiourea between intra- and extra-erythrocyte water was determined in the usual manner (see Chapter XV, Section 1). Table 4.14 shows that the non-electrolyte was apparently more concentrated in the former than in the latter, and that the two concentrations were in direct proportion. The permeation of thiourea cannot be analysed in terms of the simple theory elaborated in Part Two.

Three possible explanations for the apparent accumulation were seriously entertained, as follows.

(1) The accumulation is an experimental artifact caused by two factors. Firstly, thiourea reduces an apparent concentration of inulin by a fraction which increases with the concentration of thiourea (see pp. 123-124). Secondly, the ratio of the amount of thiourea to that of inulin in a mass of packed cells cannot be less than the ratio of the amounts of the two compounds in the supernatant. In consequence, the volume of extracellular medium in the mass of cells is underestimated, and the amount of intracellular thiourea is overestimated.

Two arguments were arrayed against this explanation. The first is that  $f'$  was independent of the concentration of extracellular thiourea,  $x$ . The explanation predicts that it should have increased with  $x$ . The second is that a correction was made for the effect of thiourea on an estimate of the concentration of inulin. Nevertheless,

the percentage recovery of inulin was generally less than that of thiourea (see Table 4.14). The explanation cannot be dismissed out of hand.

(2) Thiourea forms complex ions with both  $\text{Na}^+$  and  $\text{K}^+$  (Boeyens & Herbstein, 1966). If the complex ions had different dissociation constants, it follows that when the concentrations of intracellular and extracellular free thiourea were equal the concentrations of total thiourea would be different.

This explanation was shown to be untenable. In one experiment, the extracellular NaCl was replaced by an equimolar concentration of KCl. After 30 min, the concentration of extracellular thiourea was 20.1 mM, and the mean and standard error of  $f'$ , based on four determinations, was  $1.49 \pm 0.03$ . Since in this experiment the concentrations of intracellular and extracellular  $\text{K}^+$  were both 130 mequiv./l,  $f'$  should have been close to unity.

(3) Thiourea is bound by some other cellular constituent. Haemoglobin is an obvious candidate, because it is present in high concentration. The validity of the explanation was investigated by equilibrium dialysis.

A haemolysate of the washed erythrocytes was prepared by diluting 5 ml of the packed cells with 20 ml of water and then 25 ml of suspension medium. A 10 ml aliquot of the haemolysate, representing 4.4  $\mu\text{mol}$  of haemoglobin, was dialysed at  $25^\circ\text{C}$  against 30 ml of 60%

(v/v) suspension medium, containing 11.8  $\mu$ mol of thiourea. In a control experiment, the 10 ml of haemolysate were replaced by an equal volume of 60% (v/v) suspension medium. After 16 h, duplicate estimates were made of the concentrations of thiourea outside the two dialysis sacs. Their means were:

"experimental": 287  $\mu$ M ; "control": 289  $\mu$ M.  
It was inferred that the haemolysate had not bound any thiourea.

Therefore the reason for the apparent accumulation was not found. An adequate explanation must account not only for the independence of  $f'$  and  $x$ , but also for the following observations, which were made on cells that had been equilibrated with thiourea in the absence of an exogenous source of energy:

- (i) the volume of the cells was equal to their isosmolal volume;
- (ii) the accumulation was reversible, in that  $f'$  did not change when the cells were incubated in a medium that was free of thiourea;
- (iii) the cells appeared normal when they were examined under a light microscope;
- (iv) the "thiourea blank" of a haemolysate was zero (see p. 142).

Until an adequate explanation is found, it is reasonable to treat all data on the permeability of cells to thiourea with reserve - especially as this non-electrolyte also appears to be bound by some cellular component of Escherichia coli (A.K. Solomon, personal communication).

Chapter XVIII

SUMMARY

1. At equilibrium, the ratio of the concentrations of intracellular to extracellular pentose was unity.
2. The erythrocytes were penetrated more rapidly by b - glucose than by a - glucose.
3. The kinetics of permeation of each aldose are consistent only with the Simple Membrane Carrier Hypothesis.
4. At any one temperature between  $2.0^{\circ}\text{C}$  and  $25.5^{\circ}\text{C}$ , the aldoses had the same value of  $V$  but different values of  $K$ .
5. These values are consistent with the postulate that the aldoses share a common carrier, for which  $k_3 < k_2$ . They give no information on whether or not the free and complexed carrier have the same rate constants for crossing the membrane.
6. The values do not agree with those of other workers. Possible explanations for the discrepancy are considered, but no firm conclusion is reached.
7. The fragility of the washed cells increased when they were incubated in the presence of anti-insulin serum. Therefore their permeability to glucose could not be determined.
8. At equilibrium, the ratio of the concentrations of intracellular to extracellular meso-erythritol was unity.
9. The kinetics of permeation of erythritol are consistent only with the Simple Membrane Carrier Hypothesis.
10. The value of  $V$  for erythritol is some fifty-fold less than the corresponding value for the aldoses. This suggests that erythritol and the aldoses do not share a common carrier.



11. At equilibrium, the ratio of the concentrations of intracellular to extracellular thiourea was greater than unity. No explanation of this observation was found. Therefore the permeation of thiourea could not be analysed in terms of any of the hypotheses.

PART FIVE

GENERAL DISCUSSION

### Preamble

The present investigation into the permeability properties of the human erythrocyte falls naturally into three parts. The first is the description of the three principal hypotheses of non-electrolyte permeability, and the derivation from each of the equation defining the time-course of net permeation. In the second part, a novel technique for determining a time-course is introduced. Thirdly, the time-courses of permeation of five non-electrolytes are determined, and discussed in terms of the three hypotheses.

In this General Discussion, the main conclusions drawn in the three parts are in turn summarised and considered in the context of the permeability properties of cells in general. Some of the ways in which the investigation might usefully be extended are also indicated.

### Theory

The three principal hypotheses of non-electrolyte permeability require no re-introduction. A unique permeation equation can be derived from each of them, provided that certain assumptions hold. Thus, for the Simple Diffusion and the Simple Membrane Carrier Hypotheses, the permeant must enter the cell at a rate which is slow compared with that of water, and the cell itself must be a "perfect osmometer".

Although the first assumption is justified in this investigation, the second is not. Nevertheless, it is argued that the anomalous osmotic behaviour of the erythrocyte makes but a trivial difference.

On the other hand, the permeation equation based on the Bulk Diffusion Hypothesis is only an approximation.

Whereas an explicit equation can be derived for a sphere, an infinitely long cylinder, and a disc of infinite radius, no such equation exists for a biconcave disc of finite dimensions. Furthermore, it is not possible to allow for the change in cell volume that must accompany net permeation. It is concluded that the disc resembles the erythrocyte more closely than does the sphere or the cylinder, and that time-courses are best studied in conditions such that the change in erythrocyte volume is minimal. However, it is apparent that the permeability of, for example, a chain of muscle cells is more amenable to analysis in terms of Bulk Diffusion than is that of the erythrocyte.

Methods are given for fitting the permeation equations to an observed time-course, and for assessing

the goodness of the fit. If an equation fits a time-course, the hypothesis on which it is based appears to explain the observed kinetics of permeation, and the parameters that characterise the hypothetical rate-limiting step can be estimated.

This approach suffers from an important qualification. Although the three permeation equations seem to be different, in certain circumstances two of them may fit a given time-course. But it is shown that when these two equations are fitted to time-courses which were determined at different concentrations of extracellular permeant, only one of the sets of characteristic parameters can be constant. Therefore the ambiguity is resolved. There are, in fact, three possible ambiguities.

(1) When the time-course is consistent with both the Simple Diffusion Hypothesis and the Membrane Carrier Hypothesis (with  $x \ll K$ ). The latter hypothesis can only be excluded after time-courses have been determined at higher concentrations, and in the presence of possible competitors, of the permeant. On the other hand, if these time-courses yield a finite value of  $K$ , or if permeation is inhibited by the possible competitors, the Simple Diffusion Hypothesis must be abandoned.

(2) When the time-course is consistent with the Bulk Diffusion and the Membrane Carrier Hypotheses. If the diffusion coefficient,  $D$ , is independent of concentration, whilst  $K$  and  $V$  depend upon it, permeation is probably limited by the rate of diffusion of the

intracellular permeant.

(3) When, as in (2), the time-course is consistent with both the Bulk Diffusion and the Membrane Carrier Hypotheses, but  $K$  and  $V$ , rather than  $D$ , are independent of concentration. This could conceivably indicate a process of bulk diffusion if, for example, the diffusing molecule were the monomeric form of a compound that polymerised in aqueous solution. However, it is more likely that permeation is carrier-mediated.

A consequence of the possible ambiguities is that the classical studies of Collander & Bärklund (1933), and of Jacobs et al. (1935), must be treated with circumspection. These authors generally assumed that permeation occurs by simple diffusion; however, it is feasible that their results could be better explained in terms of permeation by facilitated diffusion. Similarly, the work of Fenichel & Horowitz (1963), and of Ling (1966), can be criticised on the grounds that although the kinetics of permeation they observed are consistent with the Bulk Diffusion Hypothesis, they have not proved that the diffusion coefficients are independent of concentration.



### The Novel Technique

The novel technique is elaborated in Part Three. It can be used to investigate the permeability properties of cells in suspension. It does not depend on changes in cell volume (c.f. the photometric technique); nor does it employ a reagent that inhibits permeation. Therefore it can be applied to a wide range of cell types and permeants.

The technique has three main limitations. It depends on an unexceptional determination of extracellular volume. The methods for estimating this volume, and the concentration of permeant, must be precise. And the technique cannot be used to study rapid permeation.

### The Present Results, and Future Work

The main conclusions of this investigation, that the aldoses and erythritol permeate the human erythrocyte by mechanisms of facilitated diffusion, agree with current dogma. However, the minutiae of the mechanisms remain to be elucidated. They may continue so, until the amount and the properties of intracellular mutarotose have been established; and until the initially heterogeneous population of erythrocytes has been fractionated into more homogeneous sub-populations. For example, the cells can be separated into classes of different density, and consequently of different age, by density - gradient centrifugation (Winterbourn & Batt, 1970).

There is no strong evidence to support the contention that the cytoplasm of the erythrocyte in any way limits the rate of permeation. A further argument against the contention was afforded by LeFevre (1961b), when he showed that the permeability to glucose of intact erythrocytes, and of their ghosts, were substantially the same.

Nevertheless, in other types of cell the plasma membrane may not be the only structure that governs permeation. For example, the data of Fenichel & Horowitz (1963) on the kinetics of efflux of several non-electrolytes from frog skeletal muscle are not compatible with the idea that the sole rate-limiting step is the diffusion, either simple or facilitated,

of the non-electrolytes across the plasma membrane. The pair concluded that the rates of efflux were limited by the slow diffusion of the non-electrolytes through the interior of the cell. But it is shown in Appendix B that if the cell comprised an outer and an inner compartment (the former representing the cytoplasm, and the latter the sub-cellular particles), and if a non-electrolyte crossed the membrane of the inner compartment by simple diffusion and that of the outer compartment by facilitated diffusion, then the time-course of efflux would be similar to those observed by Fenichel & Horowitz. It is also shown that if the efflux were measured at a different concentration of non-electrolyte, the form of the time-course would alter. Since on the Bulk Diffusion Hypothesis the form of a time-course is independent of concentration, the two mechanisms can be distinguished. Fenichel & Horowitz appear to have studied the efflux of most of their non-electrolytes at only one concentration. Therefore these data are also compatible with the cell's being a two-compartment system.

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It may well be naïve to suppose that a syncytium of muscle cells comprises a two-compartment system, as opposed to a multi-compartment one. It is easy to prove that as the number of intracellular barriers to free diffusion increases, the kinetics of intracellular diffusion approximate more closely to those of bulk diffusion. Such barriers could be regions of ordered water as well as membranes. In consequence, if a

cell had a high degree of internal organisation, its rate of permeation could be limited by diffusion through its interior as well as across its plasma membrane.

Thus the comparative simplicity of a donor's erythrocytes may favour the making of unexceptional conclusions about the step that limits a rate of permeation. Furthermore, although erythrocytes form a heterogeneous population, there is no indication that the permeability to aldoses of erythrocytes from different donors are qualitatively different. However, Laris (1967) has separated cattle in two classes on the basis of the permeability of their erythrocytes to D (-) fructose. One class has cells whose permeability is low, and is inhibited by D (+) glucose. The other has cells whose permeability is greater, and is unaffected by glucose. Moreover, in the latter cells uphill transport of labelled fructose can be induced by unlabelled fructose, but not by glucose (P.C. Laris, personal communication). These results suggest that fructose enters the erythrocytes of both classes by facilitated diffusion, but that the mechanisms are qualitatively different. The data in Appendix C, which refer to a herd of Jersey cows, support Laris' finding. Therefore it should be possible to characterise further the kinetic properties and the genetics of the two mechanisms. Indeed, it may also be possible to test the notion that the two types of carrier are correlated with different antigens on the cell surface.

Since this investigation provided no data on the

permeability of the erythrocyte to thiourea, none of the compounds examined could be said to diffuse passively through the lipid of the membrane. If such a compound could be found, the temperature - dependence of the permeability coefficient near the transition temperature of the phospholipids in the membrane might give information about the physical state of the phospholipids in this temperature range, and about the way in which the non-electrolyte diffuses through them. This consideration merely underlines the truism, explicitly stated in the General Introduction, that the study of the permeability properties of membranes can give useful insights into the relationship of cell structure and function.

APPENDICES    AND

REFERENCES



# APPENDIX A

## A Method for Inverse Non-Linear Interpolation

Suppose that  $g(x)$  is a function of  $x$ , and that its value is known at four equispaced values of  $x$  :

$(x-n), x, (x+n), (x+2n)$ . Fisher & Yates (1938) state that a good approximation to  $g(x+\theta)$ , when  $0 < \theta < n$ , is:

$$g(x+\theta) \approx \frac{1}{2} \left( \left( 2 + \frac{\theta}{n} \phi \right) \cdot g' - \frac{\theta}{n} \phi \cdot g'' \right) \quad (\text{A.01})$$

where  $g' = g(x) + \frac{\theta}{n} (g(x+\theta) - g(x))$

$$\phi = 1 - (\theta/n)$$

and  $g'' = \frac{1}{3} \left( \left( 1 + \frac{\theta}{n} \right) \cdot g(x+2) + (1 + \phi) \cdot g(x - 1) \right)$

Equation (A.01) can be re-arranged to:

$$G(\theta/n) = a(\theta/n)^3 + b(\theta/n)^2 + c(\theta/n) + d = 0 \quad (\text{A.02})$$

where  $a = 3( g(x+1) - g(x) ) - ( g(x+2) - g(x-1) )$

$$b = 3( g(x) - g(x+1) )$$

$$c = ( g(x+2) + 2 \cdot g(x-1) ) - 3( 2 \cdot g(x+1) - g(x) )$$

$$d = 6( g(x+\theta) - g(x) )$$

Equation (A.02) is solved iteratively for  $(\theta/n)$  by the Newton-Raphson process (Hartree, 1952):

$$(\theta/n)_{i+1} = (\theta/n)_i - ( G(\theta/n)_i / G'(\theta/n)_i )$$

$$= \frac{2a(\theta/n)_i^3 + b(\theta/n)_i^2 - d}{3a(\theta/n)_i^2 + 2b(\theta/n)_i + c}$$

taking  $(\theta/n)_1 = ( g(x+\theta) - g(x) ) / ( g(x+1) - g(x) )$ .

Hence, if  $g(x-n), g(x), g(x+\theta), g(x+n), g(x+2n)$  and  $n$  are all known,  $\theta$  can be computed.

Table A.01

The Accuracy of the Method for Inverse Non-Linear Interpolation

$10^3 \cdot \theta_t$	$10^3 \cdot \theta_c$	$10^3 \cdot \theta_t$	$10^3 \cdot \theta_c$
1.000	0.999	6.000	5.998
2.000	1.998	7.000	6.999
3.000	2.998	8.000	7.999
4.000	3.998	9.000	8.999
5.000	4.998	9.500	9.500

$\theta_t$  and  $\theta_c$  are, respectively, the true and computed values of  $\theta$  in  $-\ln(0.11 + \theta)$ , with  $0 < \theta < 0.01$ .

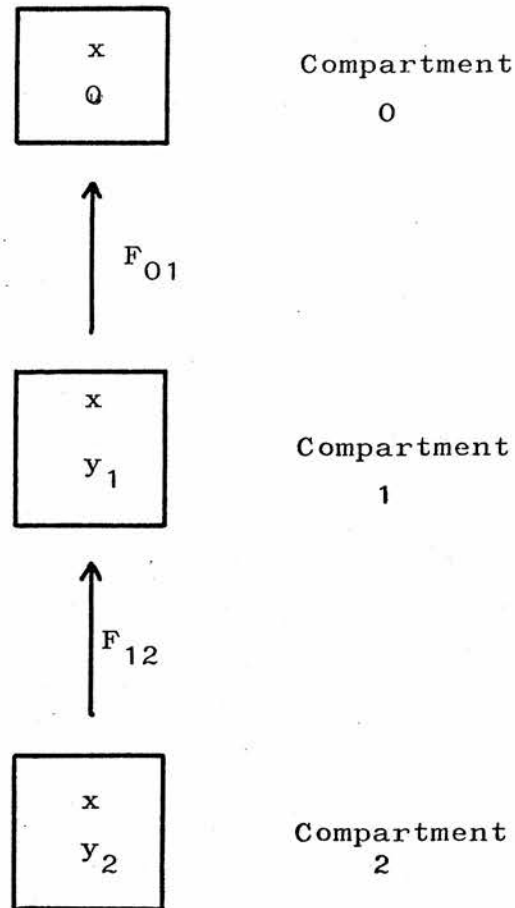
$\theta_c$  was computed from values to 5 decimal places of  $-\ln(0.10)$ ,  $-\ln(0.11)$ ,  $-\ln(0.12)$ ,  $-\ln(0.13)$  and  $-\ln(0.11+\theta)$ .

An Olivetti "Programma 101" was programmed to execute the whole computation automatically. The accuracy of the method, when executed in this way, is illustrated by the following example.

The value of  $\theta$  ( $0 < \theta < 0.01$ ) was computed from the values to 5 decimal places of  $-\ln(0.10)$ ,  $-\ln(0.11)$ ,  $-\ln(0.12)$ ,  $-\ln(0.13)$  and  $-\ln(0.11 + \theta)$ , which are given by Fisher & Yates. The true and the computed values of  $\theta$ ,  $\theta_t$  and  $\theta_c$ , respectively, are presented in Table A.01. Although the function  $-\ln(x)$  is marked curvilinear when  $0.10 \leq x \leq 0.13$ , it is clear that there is little difference between  $\theta_c$  and  $\theta_t$ .

Figure B.01

A Model of a Two-Compartment Cell



The concentration of unlabelled non-electrolyte in each compartment is  $x$ .

The concentrations of labelled non-electrolyte in compartments 0, 1 and 2 are  $x$ ,  $y_1$  and  $y_2$ , respectively.

# APPENDIX B

## The Efflux of Non-Electrolyte from a Two-Compartment Cell

Suppose that the cell is a simple two-compartment system like that depicted in Figure B.01. Compartment 1 corresponds to the cytoplasm, and compartment 2 corresponds to the sub-cellular particles. Non-electrolyte is transferred between compartment 0 (the extracellular medium) and compartment 1 by a carrier-mediated process with parameters  $K$  and  $V$ ; and between compartments 1 and 2 by simple diffusion, with parameters  $P$  and  $A$ .

In a typical experiment by Fenichel & Horowitz (1963), the cell is equilibrated with an extracellular medium containing unlabelled non-electrolyte at a concentration of  $x$  and labelled non-electrolyte at a concentration of  $x^*$ ;  $x \gg x^*$ . At time zero, the cell is transferred into a large volume of an extracellular medium which also contains unlabelled non-electrolyte of concentration  $x$  but no labelled non-electrolyte. The efflux of labelled non-electrolyte is monitored.

At time  $t$ , the concentrations of labelled non-electrolyte in compartments 1 and 2 are  $y_1$  and  $y_2$  respectively, and that in compartment 0 is effectively zero.  $F_{01}$ , the rate of net flux of labelled non-electrolyte from compartment 1 to compartment 0 is, because  $x \gg y_1$ :

$$\begin{aligned} F_{01} &= \frac{V}{K+x} \cdot y_1 \\ &= k' \cdot y_1, \quad \text{where } k' = \frac{V}{K+x} \end{aligned}$$

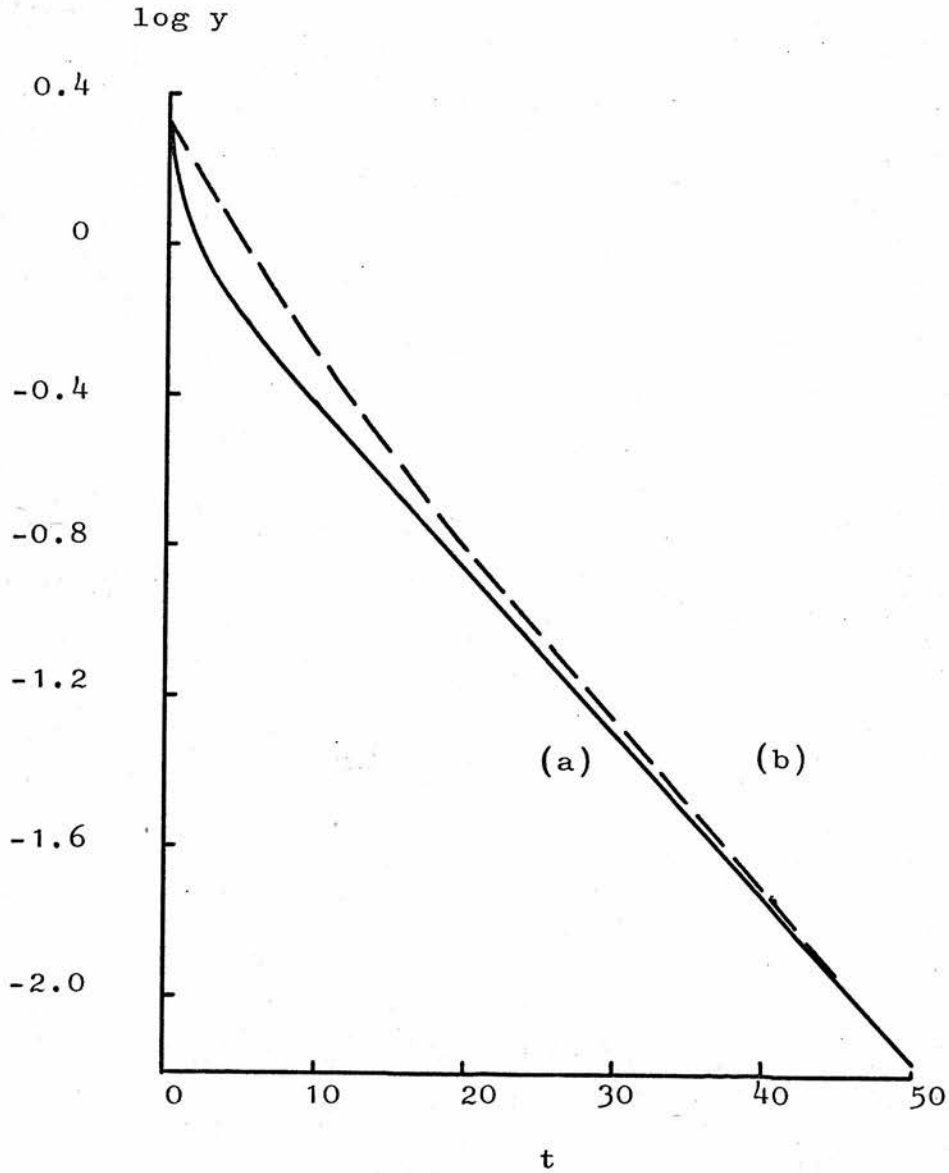
$F_{12}$ , the rate of net flux from compartment 2 to compartment 1, is

$$F_{12} = P \cdot A (y_2 - y_1) = k'' \cdot (y_2 - y_1)$$

where  $k'' = PA$ .

Figure B.02

The Time-Course of Efflux of Permeant  
from a Two-Compartment Cell



$y$  was calculated from equation (B.03), given that:

$$G = H = 1/x^*, \quad l' = 0.1,$$

and for curve (a):  $l'' = 1.0$

while for curve (b):  $l'' = 0.2$ .



$$\text{Hence } \frac{dy_1}{dt} = -k'.y_1 + k''.(y_2 - y_1) \quad (\text{B.01})$$

$$= k''.y_2 - (k' + k'').y_1$$

$$\text{and } \frac{dy_2}{dt} = -k''.(y_2 - y_1) \quad (\text{B.02})$$

Equations (B.01) and (B.02) can be solved using a Laplace transformation (Atkins, 1969). The solutions are:

$$y_1 = \frac{x^*}{1' - 1''} \cdot ((1' - 2k'').\exp(-1'.t) + (2k'' - 1'').\exp(-1''.t))$$

$$\text{and } y_2 = \frac{x^*}{1' - 1''} \cdot ((1' - k').\exp(-1'.t) + (k' - 1'').\exp(-1''.t))$$

$$\text{where } 1' + 1'' = k' + 2k''$$

$$\text{and } 1'.1'' = k'.k''$$

The mean concentration of intracellular labelled non-electrolyte,  $y$ , depends on the relative sizes of the two compartments, and is given by

$$y = x^*, (G.\exp(-1'.t) + H.\exp(-1''.t)) \quad (\text{B.03})$$

$G$  and  $H$  are positive, with  $G + H = 1$ , and are functions of  $k'$  and  $k''$ .

It follows that  $\log y$  is not proportional to  $t$ . In fact, the general form of the relationship is that shown in Figure B.02: it is similar to those observed by Fenichel & Horowitz. Its precise form depends on the values of  $k'$  and  $k''$ . In consequence, when the experiment is repeated with a different concentration of unlabelled non-electrolyte,  $k'$ , and therefore the precise form of the relationship, are altered.

## APPENDIX C

### The Permeability of Cattle Erythrocytes to Fructose

#### Introduction

Laris (1967) divided cattle into two classes, "slow" and "fast", on the basis of the permeability of their erythrocytes to D(-) fructose. "Slow" erythrocytes accumulated less than 0.25 mmol fructose /1 cells /h at 37°C from a medium of fructose concentration 4.5 mM. In identical conditions, "fast" erythrocytes accumulated between 0.7 and 2.25 mmol fructose /1 cells /h.

Laris obtained his blood samples from a slaughterhouse. This study confirms his results, for a herd of 24 Jersey cows which is maintained for research purposes.

#### Materials and Methods

A sample of blood was drawn from the jugular vein of an experimental animal into a sterile 50 ml bottle containing 50 I.U. of heparin. The sample was received within a day, and then stored at 4°C for not more than two days.

The erythrocytes were washed three times at room temperature with washing medium (125 mM Na Cl, 5 mM KCl and 10 mM Na phosphate buffer; pH at 18°C = 7.32). Their haematocrit was determined by centrifugation at 3,000 x g for 30 min. A volume of cells was diluted with washing medium to give 10 ml of a suspension of haematocrit 60%, and equilibrated with occasional manual shaking in a water bath at 38°C.

The amount of fructose taken up by the equilibrated

cell suspension in 1 h was determined as follows. At time zero, 2 ml of incubation medium (comprising 5 mg of D(-) fructose dissolved in washing medium) were rapidly pipetted into the suspension. At time 0.5 min, a 5 ml ("control") aliquot of suspension was removed, cooled in ice for 3 min, and centrifuged at  $4^{\circ}\text{C}$  and  $38,000 \times g$  for 20 min. About 2 ml of supernatant was recovered. At time 60.5 min, a second 5 ml ("experimental") aliquot of the suspension was treated identically. One millilitre of each supernatant was diluted with about 20 ml of distilled water, and deproteinised by the addition of 1 ml of 10% (w/v)  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  followed by 2 ml of 1% (w/v) NaOH. The whole was made up to a final volume of 50 ml, and centrifuged. The concentration of fructose in this supernatant was estimated using the method for the estimation of inulin elaborated in Part Three.

The amount of fructose taken up by the cells in 60 min at  $38^{\circ}\text{C}$  was calculated from the difference between the concentrations of fructose in the control and experimental supernatants. The haematocrit of the suspension being incubated was inferred from the concentration of fructose in the control supernatant. The mean concentration of extracellular fructose during the incubation was equated to the arithmetic means of the concentrations of fructose in the control and experimental supernatants.

Table C.01

The Number of Animals of Given f

$10^2 \cdot f$	No. of animals
$\leq 1.5$	0
1.6 - 3.0	3
3.1 - 4.5	9
4.6 - 6.0	3
6.1 - 7.5	2
7.6 - 9.0	0
9.1 - 10.5	0
10.6 - 12.0	5
$\geq 12.1$	2

$f$  is defined as  $y/\bar{x}$ , where

$y$  = mmol fructose taken up /1 cells /h at 38°C,

$\bar{x}$  = mean concn. of extracellular fructose, in mM.

The mean of the 24 values of  $\bar{x}$  was 4.12 mM.

## Results

The amount of fructose taken up by a sample of erythrocytes was expressed as the fraction  $f$ , defined as  $y$  (the number of millimoles of fructose taken up per litre of cells per hour at  $38^{\circ}\text{C}$ ) divided by  $\bar{x}$  (the mean concentration of extracellular fructose during the incubation, in  $\text{mM}$ ).

The distribution of the 24 animals, on the basis of their values of  $f$ , is given in Table C.01. There seem to be two distinct classes: a "low" class with an  $f$  of between  $1.6 \times 10^{-2}$  and  $7.5 \times 10^{-2} \text{ h}^{-1}$ ; and a "high" class, with an  $f$  of more than  $10.6 \times 10^{-2} \text{ h}^{-1}$ . This conclusion is reinforced by the following considerations.

(i) The "slow" class had a mean value of  $\bar{x}$  of  $4.16 \text{ mM}$ : the standard deviation of  $\bar{x}$  was  $0.01 \text{ mM}$ . The corresponding values for the "fast" class were  $4.05$  and  $0.02 \text{ mM}$ . However, because the difference between the two means is small, it is fair to compare directly the values of  $f$  of the two classes.

(ii) Twelve of the values of  $f$  are the means of determinations which were made on two samples of blood drawn at an interval of 14 days. Eleven of the animals gave duplicates which were either both "high" or both "low". The twelfth animal gave one "low" and one "intermediate" value. Therefore the distribution was reproducible.

(iii) The percentage haemolysis of the cells in a "control" aliquot had a mean of  $0.35$  and a standard deviation of  $0.20$ . The increase in percentage haemolysis on incubation averaged  $0.25$ , with a standard deviation of  $0.08$ . Therefore haemolysis cannot account for the distribution.

## Discussion

The finding of Laris (1967), that cattle can be separated into two classes on the basis of the permeability of their erythrocytes to D(-) fructose, seems to have been confirmed. But the confirmation is only qualitative. Laris' "slow" class had a value of  $f$  of about  $2.7 \times 10^{-2} \text{ h}^{-1}$ , whilst his "fast" class had values ranging from  $16 \times 10^{-2}$  to  $50 \times 10^{-2} \text{ h}^{-1}$ . Thus his "slow" and this report's "low" class are similar, whereas his "fast" class is two or three times as fast as this report's "high" class. Moreover, in Laris' experiments some of the fructose taken up by the erythrocytes may have been metabolised, so that the amount of intracellular fructose (which is what he actually estimated) was probably less than the total amount taken up.

The separation was reproducible over the period of two weeks for which the animals were examined. Moreover, there was no correlation between the class into which an animal fell and its age, the date at which it last calved, or the date at which it was due to calve next. Therefore the factors determining an animal's class may be genetic rather than environmental.

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# LIST OF SYMBOLS

The principal symbols used in this Thesis are defined below.

A	the cross-sectional area of the plasma membrane
$a', b'$	the coefficients of the regression of F on L: $F = b' \cdot L + a'$
$\alpha, \beta$	alpha -, beta -
$C, C_J$	concentration, concentration of J
$c(J)$	the coefficient of variation of J
D	the diffusion coefficient of intracellular permeant
E	extinction
e	the dimension of a cell of ideal shape (see p. 74)
F	$f'/t$
f	$y/x (= i/(x \cdot w))$
$f'$	$i/(x \cdot w_0)$
$H_J$	the blank of a haemolysate for J
i	the amount of intracellular permeant
K	the half-saturation constant of the membrane carrier (see p. 47)
$K', K''$	the estimates of K derived from the regressions of F on L, and of L on F, respectively
$k_1 \dots k_4$	the rate-constants describing the operation of the membrane carrier (see pp. 46, 166).
L	$\frac{-\ln(1-f')}{t}$
ln, log	$\log_e, \log_{10}$
m	the osmolality of the suspension medium
N	the number of molecules of carrier in a defined area of plasma membrane (see p. 47)
n	the number of observations in a time-course
P	the permeability coefficient of the plasma membrane
p, q	the coefficients of the regression of z on t: $z = p \cdot t + q$
$P_0$	the coefficient of the regression $z = p_0 \cdot t$
r	$k_3/k_4$ (see p. 166)
$s^2$	the residual variance about a regression
$s(J)$	the standard deviation or the standard error of J

# LIST OF SYMBOLS

T	transmittance
%T	per cent transmittance
t	time
V	the maximum rate of carrier-mediated permeation (see p. 47)
V', V''	the estimates of V derived from the regressions of F on L, and of L on F, respectively
v <sub>o</sub>	the volume of erythrocytes at isosmolality
var (J)	the variance of J
w	the volume of intracellular water
w <sub>o</sub>	the volume of intracellular water at isosmolality
w'	w/w <sub>o</sub>
x	the osmolality (concentration) of extracellular permeant
y	the osmolality (concentration) of intracellular permeant
Z <sub>J</sub>	the apparent increase in the concentration of J due to the presence of another compound
z	Dt/e <sup>2</sup> (see p. 74)
θ	temperature
μ	micro
φ <sub>J</sub>	the osmotic coefficient of J